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PASSIVE IMMUNIZATION
AGAINST
ORAL PATHOGENS

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*To my beloved family
Pål, Jakob and Samuel*

*Även en tusenmilafärd
börjar med ett steg
Lao-tse*

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ABSTRACT

Dental caries is one of the most common infectious diseases and *Streptococcus mutans* (*S. mutans*) is considered to be the main etiological agent of dental caries. *S. mutans* possesses a variety of virulence factors that enables it to establish and initiate disease. SA I/II a 185-kDa cell surface localized protein involved in the initial attachment to the tooth pellicle and glucosyltransferases (Gtf's), a series of enzymes involved in the production of glucans have both been shown to be important factors in the pathogenesis of dental caries. The effects of passive immunization against *S. mutans*, has previously been explored using topical oral administration of antibodies derived from both animals and plants. Lactobacilli have previously been used to deliver vaccine components for active immunization *in vivo* and are well recognized for their health promoting effects in the oral and gastro-intestinal tract. We have constructed a series of expression vectors where single-chain (scFv) antibody fragments, directed against the SAI/II antigen of *S. mutans*, are expressed on the surface of *Lactobacillus casei*, anchored or secreted. *In vitro* we verified the expression of functional scFvs and *in vivo* could show that when administered to rats, the development of dental caries was lower in comparison to controls. Furthermore, we constructed a second set of vectors containing a constitutive promoter region giving rise to a continuous expression of scFv, directed against the same SAI/II antigen of *S. mutans*. The continuous expression of scFv was compared to the previous inducible lactobacilli construct and was found to be protective against caries *in vivo* and the anti-cariogenic effect was slightly better than the effect obtained with the initial lactobacilli constructs. We have also demonstrated that polyclonal chicken derived antibodies against Gtf were protective against dental caries when administered in the drinking water to desalivated rats an important step verifying that antibodies were protective in a dry-mouth environment. Llama, dromedaries and camels produce IgG antibodies devoid of light chains and consisting of only the heavy chain part of the immunoglobulin molecule. Llama VHHs (heavy chain variable fragment) directed against the SAI/II antigen of *S. mutans*, fused with glucose oxidase, were constructed and produced in yeast. Rats receiving a daily oral dose of llama VHH or VHH-GOx had a lower development of caries. From the experiments performed herein, we have been able to establish a safe, local, and cost-efficient passive immunotherapy against dental caries.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. Weiner C*, Pan Q, Hurtig M, Borén T, Bostwick E, Hammarström L.
Passive immunity against human pathogens using bovine antibodies
Clin Exp Immunol 1999; 116:193-205.
- II. Krüger C, Hu Y, Pan Q, Marcotte H, Hultberg A, Delwar D, van Dalen PJ, Pouwels PH, Leer RJ, Kelly CG, van Dollenweerd C, Ma JK, Hammarström L.
In situ delivery of passive immunity by lactobacilli producing single-chain antibodies.
Nature Biotechnol. 2002, 20, 702-706
- III. Krüger C, Pearson SK, Kodama Y, Vacca Smith A, Bowen WH, Hammarström L.
The effects of egg-derived antibodies to glucosyltransferases on dental caries in rats.
Caries Res. 2004, 38, 9-14
- IV. Krüger C, Hultberg A, Marcotte H, Frenken LGJ, Hammarström L.
Passive immunization against caries using llama derived VHH fragments against *Streptococcus mutans*.
Submitted
- V. Krüger C, Hultberg A, Marcotte H, Ma JK, Hammarström L
Passive immunization by lactobacilli expressing single-chain antibodies against *Streptococcus mutans*.
Manuskript

* Previous name

ABBREVIATIONS

Ab	Antibody
AgI/II	Antigen I/II
AgIII	Antigen III
Bp	Base pair
CA	Cell associated Gtf
CF	Cell free
CAT	Catalytic domain of glucosyltransferase
CT	Cholera toxin
CTB	B-subunit of cholera toxin
ELISA	Enzyme-linked Immunosorbent Assay
GLU	Glucan binding domain of glucosyltransferase
FCA	Freunds complete adjuvans
FIA	Freunds incomplete adjuvans
GOx	Guucose oxidase
Gtf	Glucosyltransferase
Ig	Immunoglobulin
MDP	Muramyl dipeptide
ND	Not detected
PAc	Protein antigen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	peptidoglycan from <i>S. sobrinus</i>
SAI/II	Surface adhesion antigen
SBR	Saliva binding region of AgI/II
SC	Secretory component
ScFv	Single chain variable fragment
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electro microscopy
VHH	Variable heavy chain from llama
WSF	Water soluble fraction

INTRODUCTION

A constant dynamic equilibrium exists between the indigenous micro flora and the host defence mechanisms. The indigenous micro flora plays an important role in health and disease, and adherence to mucosal surfaces has been shown to be of great importance in the pathogenesis of various infections in the gastro-intestinal tract. In the oral cavity, adhesion to the tooth surface and oral mucosa is important and plays a role in colonization of both *Candida albicans* (*C. albicans*) and *Streptococcus mutans* (*S. mutans*). Adherence is essential for providing resistance to the flow of saliva and is mediated by adhesins on the surface of bacteria and by receptors on the oral surfaces.

DENTAL CARIES

Dental caries is one of the most common bacterial infections in humans. Although several methods, such as topical or systemic use of fluorides, fissure sealants, and dietary control, have been developed to prevent dental caries, the efficacies of these methods are not enough to eradicate dental caries in humans. In Sweden, like in other industrialized countries, there has been a decline in prevalence during the last decades of dental caries among children and adults, but still a rather large and unchanging group of individuals are suffering from severe caries (Hugoson et al., 2000a, b). Epidemiological data clearly show an increasing number of elderly people with a greater need of dental care (coronar and root surface caries) (Hugoson et al., 2000). A major reason for the decline is the massive program of prophylaxis in dental health, including information about diet, flouride and oral hygiene (Sundberg 1996). In addition, fluoride supplemented toothpastes were introduced during the 1960's and their use expanded significantly during the 1970's and contributed to the prevention of dental caries (Tvetman et al., 2003).

The character of the dental caries disease has changed from being evenly distributed within the population, to affect groups of individuals found in low-socio-economic and immigrant populations throughout Europe and elsewhere, that suffer from a more severe form of caries (Curzon et al., 2004). Although the incidence of caries has been reduced there are also other groups of patients for whom caries remains a major clinical problem. These include individuals with hypo salivation due to adverse effects of certain pharmaceutical products, systemic diseases such as Sjögren's syndrome, and head and neck tumor patients receiving heavy local irradiation therapy, may all suffer

from severe caries as a consequence a reduction in salivary flow (Spak et al., Brown et al., 1978). Patients with reduced salivary secretion, such as Sjögrens syndrome, harbours more caries associated bacteria such as mutans streptococci and lactobacilli than healthy controls (Almståhl and Wikström 1999, 2001).

Dental caries is a complex and multifactorial disease, and the development of caries is thought to result from the interplay between the host, the diet and the endogenous micro flora. Some degree of prophylaxis against caries is possible by continuous administration of fluoride, which increases the ability to remineralize early stages of the disease. It also enhances the ability to withstand a cariogenic attack and may furthermore reduce the ability of the bacteria in dental plaques to utilize the sucrose for acid production.

The specific and non specific defence in saliva

The environment of the oral cavity is controlled primarily by saliva and the continuous flow of saliva is removing a large number of microorganisms from the teeth and the mucosal surfaces. Saliva also contains several specific and non-specific defence factors. SIgA is the principal immunoglobulin isotype found in saliva and all other secretions, and consists of two (or more) IgA monomers (300 kDa), a J (joining) chain (15,6 kDa), and a secretory component (SC) (70 kDa) (Brandtzaeg et al., 1995, Kerr et al., 1990). SIgA is considered to be the main specific defence mechanism in the oral cavity and its biological functions include neutralization of enzymes, toxins and virus, or by acting in synergy with other non-specific antibacterial factors such as lysozyme, lactoferrin, salivary peroxidase and mucins. One of the major intriguing questions concerning the role in oral microbial ecology is whether sIgA can affect the indigenous bacteria. Despite high levels of sIgA in the saliva the resident micro flora still persist in the oral cavity.

Saliva also possesses defence factors with direct antimicrobial activity *in vitro*. A group of salivary proteins, lysozyme, lactoferrin, and peroxidase, act in conjunction with other components of saliva to limit the growth of bacteria or kill them directly. Lysozyme is a small cationic protein, present in all major body fluids, and lyses bacteria by hydrolysing glucosidic linkages in the cell wall peptidoglycan. It may also cause lysis by interaction with thiocyanate, perchlorate, iodide, bromide, bicarbonate, nitrate, and fluoride, and with proteases found in saliva (Mandel, 1987, Marcotte and Lavoie,

1998) *In vivo*, an inverse correlation has been found between concentration of lysozyme and the accumulation of dental plaque (Marcotte and Lavoie, 1998).

Lactoferrin is an iron-binding glycoprotein and inhibits microbial growth, probably by sequestering iron in the environment. In addition, iron-free lactoferrin (apolactoferrin) possesses a direct, iron-independent bactericidal effect against various oral bacterial strains including *S. mutans* (Marcotte and Lavoie, 1998).

Salivary peroxidase is an enzyme secreted by salivary gland acinar cells. It is part of an antimicrobial system that involves the oxidation of salivary thiocyanate to hypothiocyanite and hypothiocyanous acid by hydrogen peroxide, generated by oral bacteria. Salivary peroxidase removes toxic hydrogen peroxide produced by oral microorganisms and can reduce acid production in dental plaque (Marcotte and Lavoie, 1998). Salivary peroxidase has been shown to retain activity when adsorbed on hydroxyapatite and so should be effective at the enamel-plaque interface (Tenovuo et al., 1977). The activation of peroxidase systems *in vivo* reduces plaque accumulation, gingivitis and caries (Marcotte and Lavoie, 1998).

Streptococcus mutans

Among oral bacteria, mutans streptococci have been implicated as causative agents of dental caries. The major route for early acquisition of mutans streptococci is vertical transmission from mother to child (Aaltonen et al., 1990, Berkowitz et al., 1993). Based on DNA homology, mutans streptococci are divided into seven species: *S. mutans*, *S. sobrinus*, *S. rattii*, *S. criceti*, *S. downei*, *S. ferus*, and *S. macacae*, which can be subdivided into eight serotypes: a, b, c, d, e, f, g and h. Of these species, *S. mutans* and *S. sobrinus* have been implicated as the primary causative agents of dental caries in humans (Loesche et al., 1986).

The genome of serotype c *S. mutans* strain UA159 has been sequenced and is composed of 2,030,936 bp (base pairs) (Ajdic et al., 2002). The genome analysis provides further insight into how *S. mutans* has adapted to surviving the oral environment.

ANTIGENS OF *S. MUTANS*

A key to understand how *S. mutans* colonizes the oral cavity is discerning how the various molecular components comprising the bacterial cell surface interact with the acquired dental pellicle. In the initial studies of vaccines against dental caries, whole cells of mutans streptococci were applied systemically or locally.

S. mutans possesses various cell-surface substances, including serotype-specific polysaccharide antigens, lipoteichoic acid, glucosyltransferases (Gtfs), glucan-binding proteins, a 13 kDa protein antigen (antigen D), a 39 kDa protein (AgIII), a 29 kDa protein antigen (antigen A), a 70 kDa protein antigen (antigen C), and a 190 kDa protein (AgI/II or SAI/II) (Koga et al., 1995, Kuramitsu, 1993). These cell-surface molecules are thought to play important roles in interactions between the organism and its host, and have been given much attention as vaccine candidates against dental caries.

SAI/II surface adhesion protein, PAc or AgI/II

The *spaP* gene of *S. mutans* encodes a 185,000-*M_r* glycoprotein, referred to as streptococcal antigen SAI/II, a major cell-surface protein antigen of *S. mutans* serotype c, which has previously been implied to play a major role in colonization (Jenkinson et al., 1997). This protein has been variously designated as AgI/II, B, IF, P1, SR, MSL-1, and PAc (Koga et al., 1995, Jenkinson et al., 1997). SAI/II is an immunodominant antigen capable of eliciting both an antibody response (Lehner et al. 1981) and T-cell proliferation (Lehner et al., 1984).

The SAI/II protein possesses two internal repeating amino acid sequences; one rich in alanine (A-region), and the other rich in proline (P-region) (Fig. 1). The N-terminal domain containing the A-region has been predicted to be totally alpha-helical. The C-terminal region of SAI/II contains a potential anchor domain, which consists of a cell wall-spanning region rich in proline, a membrane-spanning region consisting primarily of hydrophobic amino acids, and cytoplasmic charged tail consisting of five charged amino acids (Murakami et al., 1997, Brady et al., 1998, Lee et al., 2000). Various salivary components, such as sIgA, β 2-microglobulin, histidine-rich polypeptides, a 60 kDa glycoprotein, lysozyme, lactoferrin, and high molecular mass glycoproteins, bind to *S. mutans* or induce agglutination of the organisms.

Isogenic mutants of *S. mutans* deficient in SAI/II cannot be aggregated in the presence of whole saliva or salivary agglutinin (Lee et al., 1989, Koga et al., 1990).

Both the ability to adhere to experimental pellicles decreases as well as the cell hydrophobicity.

Kelly et al., mapped the T-cell adhesion, and B-cell epitopes of the SAI/II and their findings suggests that the N-terminal and central regions of SAI/II bind to salivary agglutinin on enamel surfaces, and both regions are immunologically important domains.

Previous studies also suggest that the SAI/II antigen is involved in the sucrose-independent attachment and hydrophobic interactions with the complex on the tooth surface (Oho et al., 1998).

Glucosyltransferases (Gtf)

Glucosyltransferases (Gtf's) are a series of enzymes involved in the production of glucans and have since long been recognized as virulence factors in the pathogenesis of dental caries (Tanzer, 1979, Yamashita et al 1993).

Glucans, synthesized from sucrose by the Gtf of *S. mutans*, is present in human saliva and incorporated into the initial salivary pellicle formed on teeth. There they can promote selective adherence of the bacteria, which colonize human teeth (Schilling et al 1992, Steinberg et al 1993, Vacca-Smith et al 1996), and play an essential role in the accumulation of plaques.

Streptococcus mutans contains at least three *gtf* genes that encode GtfB, GtfC and GtfD. GtfD synthesizes primarily water-soluble glucans from sucrose, while GtfB is responsible for the formation of insoluble glucans. GtfC synthesizes predominantly insoluble glucans but also significant amounts of soluble glucan (Loesche 1986, Hanada et al 1989). These glucans consist of α -1, 3-linked and α -1, 6-linked glucose residues. The water-insoluble glucan contains a relatively high proportion of α -1, 3-linked glucose residues. On the other hand, the water-soluble glucan contains of α -1, 6-linked glucose residues. The protein consists of a sucrose-binding domain on the N-terminal part and a glucan-binding domain on the C-terminal part (Fig.1b).

In the specific pathogen-free rat model systems, mutants defective in *gtfB* or *gtfC*, or both, exhibit markedly reduced levels of smooth-surface carious lesions relative to the parental organism (Yamashita et al., 1993). A mutant defective in *gtfD* gene also produces significantly fewer smooth-surface lesions than the parental strain. These findings indicate that all three *gtf* genes are important for smooth-surface caries formation.

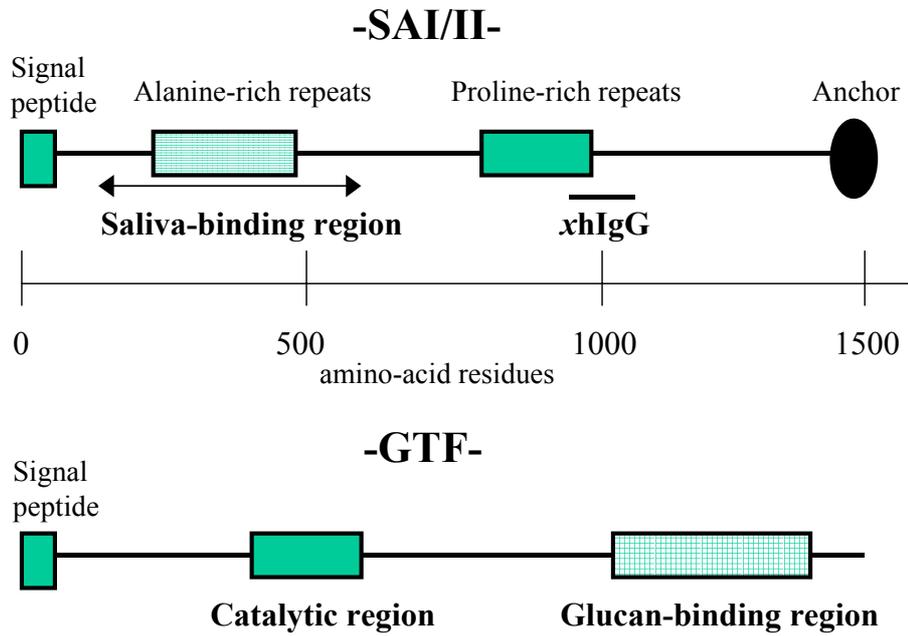


Fig. 1. The overall structural organization and localization of functional regions of SAI/II (185 kDa) and GTF-I (162 kDa)

MECHANISMS OF ANTIBODY INHIBITION

In hosts immunized with antigens from mutans streptococci, specific antibodies are induced in the saliva and the gingival crevicular fluid (Koga et al., 2002). SIgA is the predominant Ig in saliva and is considered to be the main specific defense in the oral cavity. Secretory antibodies could help maintain the integrity of the oral surfaces by limiting microbial adherence to epithelial and tooth surfaces; by neutralizing enzymes, toxins and virus; or by acting in synergy with other antibacterial factors such as lysozyme, lactoferrin, salivary peroxidase, and mucins. Antibodies directed against *S. mutans* may induce cellular aggregation and thereby decrease the number of mutans streptococci adhering to the tooth surface (Liljemark et al., 1981).

Antibodies can interfere by blocking the epitopes of the adhesins of *S. mutans*. Both polyclonal and monoclonal antibodies to SAI/II (AgI/II) and the saliva-binding region (SBR) of the antigen strongly reduce the sucrose-independent adherence of *S. mutans* cells to tooth surfaces (Brady et al., 1992, 1993).

Specific antibodies directed against the catalytic domains of enzymes should inhibit the enzyme activity and it has previously been shown that antibodies to Gtfs inhibit the activity of these enzymes (Krüger et al., 2004) and the synthesis of glucans (Taubman et al., 1995). Previous investigations reveal that antibodies against ribosomal preparations from *S. sobrinus* inhibited glucose transport by the phosphotransferase system, acid production from sucrose, and growth of the organism (Gregory et al., 1984). Moreover, bovine antibodies directed against whole cells of *S. mutans* were able to inhibit the uptake of glucose in the bacteria (Loimaranta et al., 1998).

It has also been shown that antibodies directed against *S. mutans* antigens can stimulate phagocytosis and killing by polymorphnuclear leukocytes (Lehner et al., 1985, van Raamsdonk et al., 1996, Loimaranta et al., 1999).

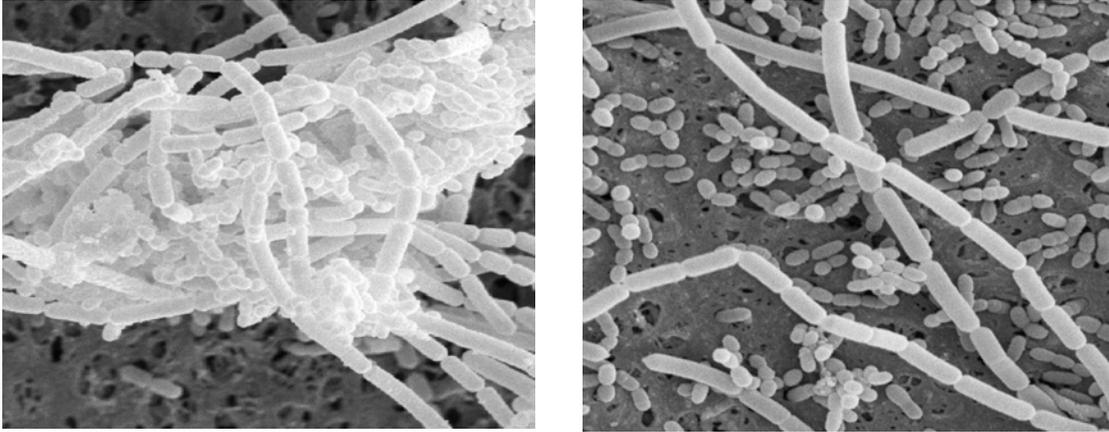


Figure 2. Showing the mechanism of agglutination between transformed *L. zeae* (*L. casei*) expressing scFv directed against SAI/II and *S. mutans* Ng8 (expressing the epitope of SAI/II). The second picture is the transformed *L. zeae* empty vector control and *S. mutans* Ng8.

Adsorption of antibodies on the cell surface might influence the physicochemical surface of the mutans streptococci to turn from hydrophobic to more hydrophilic thereby reducing the ability to adhere to the tooth surface (van Raamsdonk et al., 1995).

It has also been shown that when *S. sobrinus* was incubated during growth with antibodies, the morphology on the micro colonies changed to become fine-meshed and form long chains of bacteria. These bacteria are more easily removed from the oral cavity and have reduced pathogenic potential (van Raamsdonk et al., 1997).

ACTIVE IMMUNIZATION AGAINST DENTAL CARIES

Experimental vaccination against *S. mutans* has been shown to result in a high level of specific salivary antibodies, which correlate with prophylaxis against caries development. Numerous attempts have been made to improve the immune response against the bacteria using different adjuvants and various routes of vaccination (Koga et al., 2002). Using animal models, the induction of salivary IgA and serum IgG has been studied with various antigen challenges of either whole cells of *S. mutans* or purified proteins such as SAI/II, Gtfs and recombination proteins (Koga et al., 2002). Different immunization routes have been used like subcutaneous, oral, intranasal, and topical routes. Moreover, various adjuvants and delivery vehicles have also been used to enhance or modify the immune responses to mutans streptococci. They have all been able to give rise to a both mucosal and systemic response and reduce the *S. mutans* in plaque and lower the development of dental caries (Koga et al., 2002). The effects of active immunization in animal studies are presented in table 1.

In the initial studies, whole *S. mutans* bacteria were used, but the antibodies generated were found to be cross-reactive against heart muscle tissue and it has been difficult to rule out the risk of cross-reactivity (Hughes et al., 1986). Due to this risk, the development of subunit vaccines has been the focus of intense research interest.

In humans, several studies have been performed using whole cells of heat or formalin killed mutans streptococci, capsulated or non-capsulated. The immunological response was mainly in the saliva (sIgA) and reduced to some extent the colonization of mutans streptococci (Gahnberg et al., 1983, Cole et al., 1984, Gregory et al., 1987). Oral administration of *S. sobrinus* derived Gtf-I, in gelatin capsules, elicited a salivary IgA antibody response when combined with an aluminium-based adjuvant, and were able to interfere with the re-accumulation of *S. mutans*. Also, Childers et al. reported that salivary IgA1 and IgA2 anti-Gtf responses were induced in human subjects after ingestion of enteric-coated capsules containing *S. mutans* Gtf (Childers et al., 1994). The same group also showed that nasal immunization of human subjects with *S. mutans* Gtf in liposomes is effective in inducing a secretory IgA antibody response (Childers et al., 1997, 1999).

Systemic immunization is difficult to justify because of its undesirable side effects. There is evidence of cross-reacting antigens between *S. mutans* and human heart tissues, and achievement of a long lasting mucosal immunity against the indigenous

microflora is a complex issue. Also, there are no studies performed in humans showing protection against development of dental caries.

Table 1.Effects of active immunization against *S. mutans* and dental caries in animals

Animal	Immunization route	Antigen	Adjuvant	Mucosal immunity	Systemic immunity	Effects	Reference
Monkeys	Subcutaneous	AgI/II (PAc)	FIA	ND	IgG>IgM>IgA	Reduction of <i>S. mutans</i> in plaque 70% inhibition of dental caries	Lehner et al., 1992
Monkeys	Subcutaneous	Antigen A and B	Aluminum hydroxide	ND	IgG	Reduction of <i>S. mutans</i> in plaque and complete inhibition of dental caries in monkeys immunized with antigen A Some protection with antigen B	Russel et al., 1982
Monkeys	Intraoral (adjacent to salivary glands)	<i>S. ratti</i> Gtf, <i>S. mutans</i> Ftf, <i>S. mutans</i> GH	FIA	ND	ND	69, 62, and 57% reductions of carious lesions in the mutans streptococci-infected monkeys immunized with Gtf, Ftf, and GH, respectively	Bahn et al., 1977)
Hamsters	Subcutaneous	Gtf	FCA	IgA	IgG	Reduced colonization of <i>S. mutans</i>	Russel et al., 1987
Rats (conventional)	Subcutaneous (salivary glands)	Gtfs, CAT (synthetic peptides), GLU, CAT+GLU, CAT-GLU	FCA	IgA	IgG	Reduction of <i>S. mutans</i> in plaque Significant reduction of caries development	Smith et al., 1997, Taubman et al., 1995, 2000, 2001

Rats (gnotobiotic)	Parental (region of salivary glands) Oral	Anti-idiotypic vaccine	None	None	IgG	Reduction of <i>S. mutans</i> in plaque Inhibition of dental caries	Jackson et al., 1990
		Anti-idiotypic vaccine	Liposome	IgA	None	Reduction of <i>S. mutans</i> in plaque Inhibition of dental caries	
Rats (gnotobiotic)	Oral	Ribosomal proteins	Liposome	IgA	IgG (low)	Reduction of <i>S. sobrinus</i>	Gregory et al., 1986
Rats (gnotobiotic)	Oral	Whole cells and cell walls of <i>S. sobrinus</i>	MDP, PG	IgA>IgG	IgA>IgG, IgM	Reduction of <i>S. sobrinus</i> in plaque Inhibition of caries development	Morisaki et al., 1983)
Rats (gnotobiotic)	Oral (gastric)	Crude Gtf	Liposome	IgA	IgM>IgA>IgG	49-64% reduction of <i>S. mutans</i> in plaque Lower (P<0.05) caries scores on all molar surfaces compared with controls	Childers et al., 1996
Rats	Oral	Recombinant <i>S. typhimurium</i> expressing SpA of <i>S. sobrinus</i>		IgA	Ig	Reduction of <i>S. sobrinus</i> in plaque Significant (P<0.05) inhibition of caries development	Redman et al., 1996
Mice	Intranasal	PAC	CTB	IgA	IgG>IgA, IgM	Reduction of <i>S. mutans</i> colonization	Takahashi et al., 1991
		PAC (301-319)	CTB	None	IgG	Reduction of <i>S. mutans</i> colonization	
Rats (gnotobiotic)	Intranasal	AgI/II	CTB	IgA	IgG>IgA, IgM	99% reduction of <i>S. mutans</i> in plaque. 30 and 73% reduction enamel caries	Katz et al., 1993

Rats (conventional)	Intranasal	AgI/II	CTB	IgA	IgG>IgA, IgM	38% reduction of <i>S. mutans</i> in plaque 64% reduction in buccal enamel caries	Katz et al., 1993
Rats (conventional)	Intranasal	SBR-CTA2/B AgI/II or AgII	CT, CTB, +CT	IgA	IgG	Inhibition of caries development AgI/II-CTB>SBR-CTA2/B>AgII	Hajishengallis et al., 1998
Mice	Intranasal	GLU of Gtf-I, -Tio	None	IgA	IgG	Reduction of <i>S. mutans</i> colonization Inhibition of caries development	Jespersgaard et al., 1999
Rats	Intranasal	Fimbriae-enriched preparations from <i>S. mutans</i>	CTB	IgA	IgG	Reduction of <i>S. mutans</i> colonization	Fontana et al., 1999
Monkeys	Topical gingival	3.8K SA	None	IgA	Gingival IgG	75% reduction of <i>S. mutans</i> in smooth-surface plaque	Lehner et al., 1986
Monkeys	Topical gingival	Synthetic peptides from 3.8K SA	None	IgA	Gingival IgG	Reduction of <i>S. mutans</i> colonization	Lehner et al., 1989
Rabbits	Tonsillar	<i>S. sobrinus</i> cells	None	IgA	IgG	Reduction of <i>S. sobrinus</i> in plaque 75% reduction in caries development	Fukuizumi et al., 2000

FIA, Freund's incomplete adjuvant; ND, not determined, FCA, Freund's complete adjuvant; Gtf, glucosyltransferase; Ftf, fructosyltransferase; GH, glycosidic hydrolases; CAT, catalytic domain of Gtfs of mutans streptococci; GLU, glucan-binding domain of mutan streptococci, MDP, muramyl dipeptide; PG, peptidoglycan from *S. sobrinus*; CT, cholera toxin; CTB, the B subunit of CT; -CTB, coupled chemically with CTB, SBR-CTA2/B, a chimeric protein which the toxic A1 subunit of CT is replaced by an N-terminal saliva-binding region SBR of AgI/II (PAC); AgII, a C-terminal region of AgI/II; -Tio, coupled chemically with thioreoxin from *E. coli*; 3.8 kDa fragment of AgI/II.

PASSIVE IMMUNISATION AGAINST DENTAL CARIES

Local passive immunization is a safe procedure for controlling growth of microorganisms and preventing disease in the gastro-intestinal tract (Weiner et al., 1998). Passive immunization against *S. mutans* has been tried using oral administration of antibodies directed against whole cells of *S. mutans* or purified components such as the glucosyltransferase (Gtf), streptococcal antigen I/II (SA I/II) or serotype carbohydrate (Table 2.).

Bovine antibodies

Bovine antibodies are actively transported from plasma to milk in cows, and are present in high concentrations in colostrum (Weiner et al., 1998). Bovine colostrum contains 40-200 mg/ml of antibodies and have been used for passive immunization in prevention measures targeting various pathogens including *S. mutans*.

Loimaranta et al., showed that bovine immune colostrum from cows immunized with whole cells of *S. mutans* and *S. sobrinus* had a significant inhibitory effect on glucose uptake and formation of glucan and fructan by *S. mutans* (Loimaranta et al., 1997). Also, colostrum whey proteins from immunized cows inhibited the adherence of *S. mutans* to saliva-coated hydroxyapatite, promoted the cell-aggregation of mutans streptococci, and supported the phagocytosis and killing by human leukocytes (Loimaranta et al., 1997, 1999). Furthermore, mouth rinsing with this bovine immune whey by human subjects decreased the relative number of mutans streptococci (Loimaranta et al., 1999).

Michalek et al. examined the anti-cariogenic effects of whey from cows immunized with seven serotypes (a-g) of mutans streptococci (Michalek et al., 1987). They found that rats receiving diet containing whey product had lower plaque scores, fewer streptococci and reduced caries activity than rats given a diet containing control whey. In a recent study (Mitoma et al., 2002) prepared bovine milk containing antibodies against a fusion of the saliva-binding alanine-rich region of PAc with the glucan-binding domain of Gtf-I and when fed to rats, the caries development was significantly less when compared to controls.

Bovine milk has several advantages for use in humans as means of passive immunization, because it is a common food that is easily delivered to the oral cavity and produced on a large scale is possible.

Egg yolk antibodies

The concentration of IgG in egg yolk can reach 25 mg/mL (Rose, Orlans and Buttress, 1974) and is referred to as IgY. Immunoglobulins from hens immunized with whole *S. mutans* bacteria have been purified and fed to rats in a supplemented diet (0.5%). Rats receiving IgY had significantly fewer caries lesions than did control rats on a normal diet (Otake et al., 1991). Moreover, human volunteers using a mouth rinse containing chicken IgY directed against *S. mutans* decreased the percentage of *S. mutans* in saliva and plaque relative to all streptococci (Hatta et al., 1997). Chicken antibodies directed against cell-associated Gtf administered in the diet to rats inhibited the colonization of *S. mutans* and reduced dental caries development (Hamada et al., 1991). Also, short-term administration of IgY antibodies to *S. mutans* glucan-binding protein B inhibited the colonization of *S. mutans* and reduced caries development in molar teeth of rats (Smith et al., 2001).

Monoclonal antibodies

Local passive immunization by repeated application of anti-AgI/II monoclonal antibodies to the deciduous teeth of Rhesus monkeys have also been performed and prevented significant colonization of the fissures and smooth-surfaces of the teeth by *S. mutans* and the subsequent development of dental caries over a period of 1 year (Lehner et al., 1985).

In rats, local application of a monoclonal antibody reactive with antigen B (SpaA) of *S. sobrinus* reduced the colonization of implanted *S. sobrinus*, compared with controls (van Raamsdonk et al., 1993). Furthermore, Ma et al. (Ma et al., 1989, 1987) reported that topical application of monoclonal antibodies directed against AgI/II on teeth of healthy volunteers prevented colonization by *S. mutans*.

The effect of the antibodies is thought to require a bivalent structure and Fab fragments have been suggested to be largely ineffective (Ma et al., 1990), suggesting that agglutination of the bacteria may play a major role for the prophylactic effect observed.

Ma et al. (Ma et al., 1995) succeeded in cloning and expressing a murine monoclonal antibody against SAI/II in transgenic tobacco plants (*Nicotiana tabacum*). The genes encoding the heavy and light chains of the monoclonal antibody against SAI/II, a murine J chain, and a rabbit secretory component, were introduced into separate tobacco plants (Ma et al., 1995). After sexual crosses between these plants and

recombinants the result was plants that expressed all four protein chains, simultaneously. The plant secretory antibody survived longer in the human oral cavity (3 days compared to 1 day) than the parent murine IgG antibody, and protected against oral colonization by *S. mutans* for at least 4 months (Ma et al., 1998). Kelly et al., (Kelly et al., 1999) used a synthetic peptide (p1025) derived from SAI/II, and demonstrated that the peptide can prevent recolonization with *S. mutans*.

Table 2.

Effects of passive immunization against *S. mutans in vivo*

Subject	Antibody origin	Specificity of antibodies	Administration procedure	Efficacy	Reference
Monkeys	Murine MAb	AgI/II	Topical application	Decreased colonization of <i>S. mutans</i> in inhibition of dental caries development	Lehner et al., 1985
Rats	Murine MAb	SpA (antigen B)	Topical application	Reduced colonization of implanted <i>S. sobrinus</i>	Van Raamsdonk et al., 1993
Rats (SPF)	IgY	<i>S. mutans</i>	Diet	60% inhibition of total caries development	Otake et al., 1991
Rats (SPF)	IgY	Cell-associated Gtf, Cell-free Gtf, whole cells of <i>S. mutans</i>	Diet	Reduced colonization by <i>S. mutans</i> and 40% reduction of total caries development. No protection against caries with IgY to cell-free Gtf and IgY to whole cells	Hamada et al., 1991
Rats	IgY	GLU	Diet and drinking water	Inhibition of <i>S. mutans</i> colonization	Smith et al., 2001
Rats (gnotobiotic)	Bovine milk	Mutans streptococci (serotype a-g)	Diet	Inhibition of dental caries Inhibition of colonization by <i>S. mutans</i>	Michalek et al., 1987
Rats (SPF)	Bovine milk	PAcA-GB	Diet	Inhibition of caries development Significantly less caries development	Mitoma et al., 2002
Humans	Murine MAbs	AgI/II	Topical application	Decreased colonization by an exogenous strain of <i>S. mutans</i>	Ma et al., 1987

Humans	Murine MAb	AgI/II	Topical application	Inhibition of re-colonization by indigenous <i>S. mutans</i> Protection 1 year after application	Ma et al., 1989
Humans	Plant SIgA/G	AgI/II	Topical application	Specific protection of re-colonization by indigenous <i>S. mutans</i> for at least 4 months	Ma et al., 1998
Humans	IgY	<i>S. mutans</i>	Mouth rinse	Reduction in ratio of <i>S. mutans</i> per total streptococci in saliva in the 4h test using a mouth rinse containing 10% sucrose Tendency for reduction in the ratio in plaque but not saliva in the 7-day test using a mouth rinse without sucrose	Hatta et al., 1997
Humans	Bovine milk (colostral)	<i>S. mutans/S. sobrinus</i>	Mouth rinse	Reduction in relative number of mutans streptococci in plaque	Loimaranta et al., 1999
Humans	Bovine milk	PAcA-GB	Mouth rinse	Inhibition of re-colonization by indigenous <i>S. mutans</i>	Shimazaki et al., 2001

MAb; monoclonal antibody; IgY, hen egg yolk antibody; SPF, specific-pathogen-free; GLU, glucan-binding domain of Gtf; PAcA-GB, a fusion protein of the saliva-binding alanine-rich region of PAc with the glucan-binding domain of *S. mutans* Gtf-I.

POSSIBLE SIDE EFFECTS OF IMMUNIZATION

The discovery that antisera from rabbits immunized with whole cells and a high molecular weight protein antigen (IF or P1) of *S. mutans* were found to be cross-reactive with normal rabbit and human heart tissue hampered progress in this field. (Ferretti et al., 1980, Hughes et al., 1980) of caries vaccination development. However, studies have been performed claiming that immunization with purified AgI/II (Bergmeier et al., 1983, Wu et al., 1990) or recombinant segments or synthetic peptides should not elicit cross-reactive antibodies against potentially undesirable epitopes, sharing homology with the host.

As an alternative approach to prevent dental caries, local passive immunization of antibodies specific for *S. mutans* antigens has recently received much attention. Antibodies have been purified from colostrum milk and egg yolk and can be administered without inducing a systemic immunological response, thereby preventing possible cross-reactivity. Several studies have shown a long lasting protective effect in humans, up to 2 years, and it has also been suggested that cariogenic mutans streptococci may be replaced by other less cariogenic microorganisms, occupying their ecological niche (Ma et al., 1990, 1998) and thus preventing caries development.

Dental caries is not a life threatening disease and therefore, risk-free and more effective approach to prevent human dental caries should be developed. Local passive immunization is safe but still too expensive and future research should be directed towards a more cost-efficient production of antibodies.

LACTOBACILLI IN THE ORAL AND GASTRO-INTESTINAL TRACT

Lactobacilli are Gram-positive bacteria that have been used in food fermentation and preservation for centuries, and some strains are normal constituents of the human intestinal microflora (Ahrne et al., 1998). They are considered as GRAS organisms (generally regarded as safe) and are claimed to be non-pathogenic. Several authors have suggested that fermented products positively affect the course of infectious diseases such as diarrhea and inflammatory bowel disease (Shornikova et al., 1997, Gionchetti et al., 2000, Guandalini et al., 2000). Selected strains of *Lactobacillus casei* and *Lactobacillus plantarum* have also been shown to exert strong adjuvant effects on the mucosal and systemic immune response (Isolauri et al., 1995, Pouwels et al., 1996). Furthermore, lactobacilli have been suggested as carriers for oral vaccines (Pouwels et al., 1998) and expression vectors have previously been constructed for surface expression or secretion of various antigens (Pouwels et al., 1996, Shaw et al., 2000, Pouwels et al., 2001, Savijoki et al., 1997).

In the oral cavity lactobacilli have long been considered as cariogenic bacteria due, to their presence in carious dentin and production of lactic acid, promoting the progression of dental caries decay. Recently, when analyzing strains of lactobacilli collected from caries free (CF) and caries active (CA) patients, lactobacilli found in CA subjects showed greater ability to adhere to hydrophobic substances, had greater salt agglutination properties and showed lower production of inhibitory substances (Ahmuda Mdel et al., 2003).

Lactobacillus rhamnosus GG (LGG), isolated from healthy human subjects has been shown to produce substances with potent inhibitory activity on a wide range of bacteria including *Streptococcus* spp (Silva et al., 1987). It has been suggested that lactobacilli strains (LGG) may compete with streptococi for oral adherence sites (Wei et al., 2002). LGG temporarily colonizes the oral cavity and has been shown to inhibit *S. sobrinus* growth *in vitro* (Meurman et al., 1994, 1995). *Lactobacillus* strains collected from human subjects have also been shown to inhibit the formation of insoluble glucan produced by *S. mutans* strain Ingbritt (Chung et al., 2004). In studies on humans, a randomized, double blind study was performed where day-care children received milk containing LGG during 7 months. The results showed less development of dental caries in the LGG group and lower mutans streptococcus counts at the end of the study (Näse et al., 2001). Short-time consumption of LGG containing cheese did also, to some extent, decrease the number of mutans streptococci (Ahola et al.,

2002). Taken together these studies point to the possibility of using selected *Lactobacillus* strains as probiotics against dental caries.

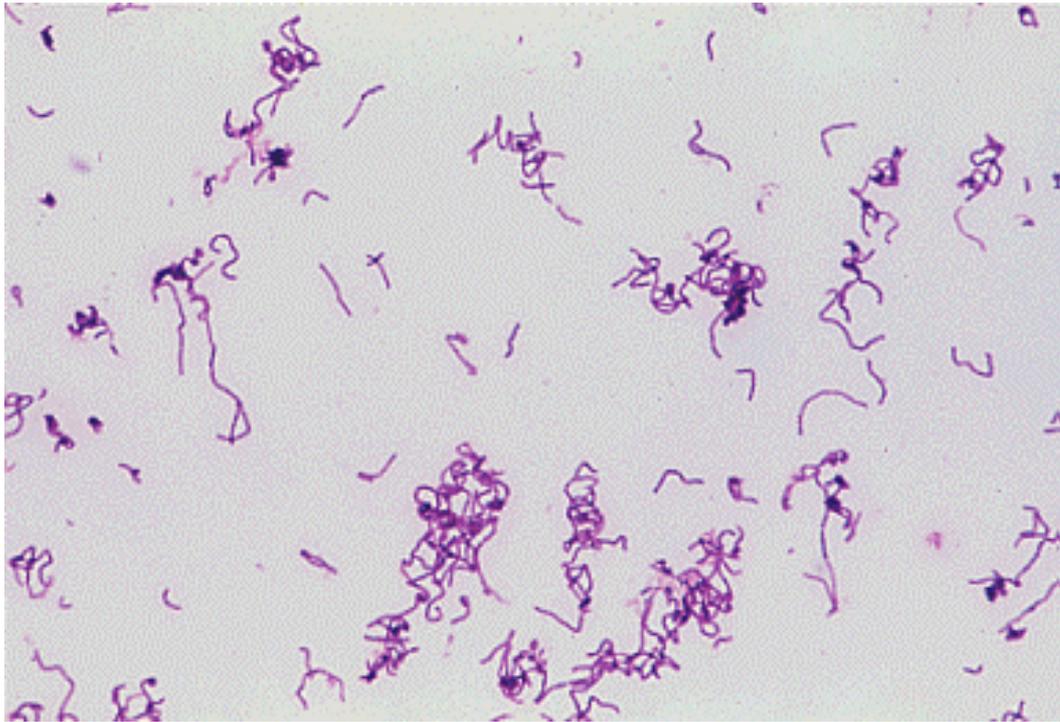


Figure 5. Show gram positive rod shaped *L. zeae*

AIMS OF STUDY

GENERAL AIM

The overall aim of this study was to develop and evaluate a safe and cost efficient passive vaccine against oral diseases

SPECIFIC AIMS

First, evaluate the anti-cariogenic effects of orally administrated chicken antibodies directed against Gtfs in a desalivated rat model, trying to determine the effect of antibodies in a dry mouth environment administered as a drinking solution.

Furthermore, evaluate the effect on dental caries development of llama VHHs directed against *S. mutans* and applied daily in the oral cavity of rats.

Second, develop a system for *in situ* expression of scFv directed against SAI/II antigen of *S. mutans* on the surface of *Lactobacillus casei*, and evaluate the function *in vitro* and *in vivo*. Also, further development and refinement of the lactobacilli constructs and evaluate the function and anti-cariogenic effects *in vitro* and *in vivo*.

MATERIALS AND METHODS

PRODUCTION AND PURIFICATION OF CHICKEN IGY

The cell-associated Gtf used for immunization was prepared as previously described (Hamada et al., 1989). Briefly, cell associated-Gtf of *S. mutans* MT8148 (serotype c) was extracted by treatment with 8 M-urea at 25°C for 1h. The crude extract was purified by DEAE-Sephacel and hydroxyapatite column chromatography. The molecular mass of CA-Gtf was determined by SDS-PAGE.

Two hundred white Leghorn hens (18 weeks old) were used and divided into two groups. The hens in group one were immunized intramuscularly with CA (cell associated)-Gtf (0.4 mg/0.5 ml) mixed with FCA (Freund's complete adjuvant 0.5 ml). The second group was immunized with an emulsion of saline (0.5 ml) and FCA (0.5 ml). After the initial immunization, two booster injections of the emulsions were given intramuscularly 7 and 13 weeks following the initial injection. Eggs were collected and kept at 4°C until used.

The preparation of antibodies and control powder from egg yolk was performed as previously described (Hamada et al., 1991). Briefly, the yolks were separated from the eggs of immunized or sham-immunized hens, mixed with saline and chloroform and incubated at 20°C for 30 min. After centrifugation, the water-soluble fraction (WSF) was separated and lyophilized. The preparation was further purified by ammonium sulfate precipitation and DEAE-Sephacel column chromatography, resulting in a product containing 98% of IgY.

CONSTRUCTION AND PRODUCTION OF LLAMA VHH IN YEAST

Young adult male llamas were injected at days 0, 21 and 35 with 250 µg of *S. mutans* in Specol (ID-DLO, Lelystad, The Netherlands) (Boersma et al., 1992). About 150 ml of blood was collected 7 days after the last immunization and peripheral blood cells sampled via Ficoll-Paque centrifugation (Pharmacia). Total RNA was isolated and after purification of mRNA (Oligotex 70022, Qiagen) and first strand cDNA synthesis (cDNA kit RPN 1266, Amersham Biosciences, Uppsala, Sweden), DNA fragments encoding VHH and part of the hinge were amplified by PCR using specific primers. The DNA fragments were ligated in the *E. coli* phagemid vector pUR4536 (Frenken et al., 2000).

The GOx protein sequence was linked through a 7-residue linker [G-S-S-G-G-S-S-] to the protein sequence of S36-VHH; this was followed by an 11-residue myc-tag [E-Q-K-L-I-S-E-E-D-L-N] to enable detection using monoclonal anti-myc antibodies (mAb). DNA fragments encoding the S36-VHH domain and the fusion protein S36-VHH-GOx were amplified by PCR (polymerase chain reaction) using specific primers, and were ligated into *Saccharomyces cerevisiae* (*S. cerevisiae*) expression plasmids, pUR 4603 containing S36-myc and pUR 4572 GOx-S36-myc. The production procedure of VHH using the yeast strain SU51 has previously been described in detail (Frenken et al., 2000).

CLONING AND EXPRESSION OF SCFV IN LACTOBACILLI

The single-chain antibody gene fragment encoding the Guy's 13 monoclonal antibody (scFv, 711 bp), was amplified from cDNA and cloned into the pCANTAB 5E phage vector (Amersham) at the *Sfi*I and *Not*I restriction sites, generating pCANT-scFv. The 3' terminus of the scFv-encoding gene was fused with an E-tag-encoding gene to allow serological detection. The scFv was amplified from the pCANT-scFv vectors using specific primers and cloned into pBluescript II SK (+) (Stratagene, La Jolla, CA) at the *Cla*I and *Eco*RI sites, generating the pBS vector. The E-tag fragment was obtained from the pCANT-scFv using primers tag-sens and pcant-antisense and was inserted at the *Eco*RI and *Sac*II sites of pBS, generating the pBSE construct.

A series of scFv expression vectors were constructed containing the promoter and secretion signal sequence of the regulated alfa-amylase gene of *L. amyolovor* as described previously (Pouwels et al., 2001). scFv was produced in *L. zea*e ATCC 393, either anchored on the bacterial surface or secreted in a free form. The scFv-E-tag fragment from the pBSE construct was amplified with two sets of primers: Sc-sense and etag-antisense A, and Sc-sense and etag-antisense B. A stop codon was introduced in the etag-antisense B primer for the construction of the secretion vector. The PCR products were subcloned into an *Escherichia coli* (*E. coli*) vector, pTUAT, generating pTUAT-A and pTUAT-B, respectively. For construction of the expression vector encoding an anchored product, the *Tldh*, the scFv, the anchor region, and the *Tcbh* were excised from pTUAT-A with *Bgl*III and *Nhe*I and cloned into pLP402 between the *Bam*HI and *Nhe*I restriction sites. The reporter gene *gusA* and subsequently, the *Tldh* were deleted from the construct generating the pLP402-scFv- short anchor vector. A construct with an elongated anchor was also made by replacing the existing anchor in

the pTUAT by the last 732 bp of the coding region of the *PrtP* gene of *L.zeae* ATCC 393. The long anchor is approximately twice the size of the short anchor (244 amino acids rep. 117 amino acids). For the construction of the secretion vector, the scFv-E-tag fragment was excised from pTUAT-B using *Bgl*III and *Xho*I and inserted between the *Bam*HI and *Xho*I restriction sites of pLP402. The Tldh was then deleted, generating the pLP402-scFv-secreted vector (Fig 4).

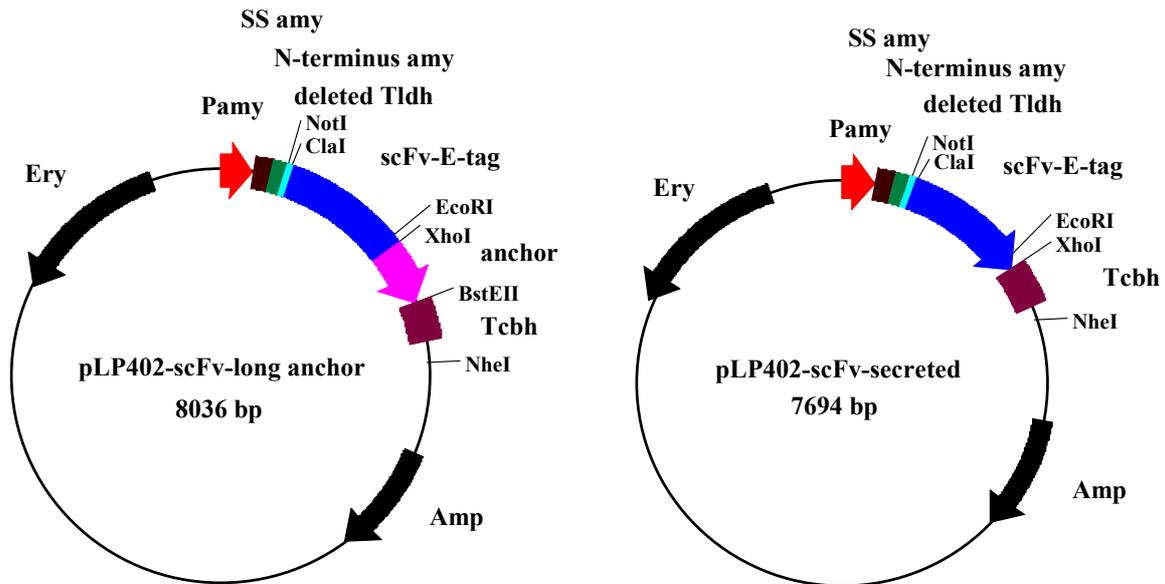


Figure 4.

Map of the pLP402-scFv-long anchor and the pLP402-scFv-secreted vector constructs

E. coli JM 109 (Stratagene) was used as the host strain for the construction of shuttle vectors and these were introduced by heat shock. Lactobacilli were transfected by electroporation at 2,5 kV, 25 μ F and 200 Ω in a Gene Pulser II (BioRad Laboratories, Hercules, CA).

For construction of the *Lactobacillus* expression vector, pLP502-scFv-long anchor, the cassette containing the *Tldh*, the *gusA* and the scFv fused to the E-tag was excised from the pTUAT-B construct at the restriction sites *Bgl*II and *Nhe*I (Promega, Madison, WI). The cassette was ligated into the *E. coli/Lactobacillus* shuttle vector pLP502 between the *Bgl*II and *Nhe*I restriction sites. The plasmid contains the constitutive promoter (*Pldh*) from the lactate dehydrogenase gene of *L. plantarum* 80 (Pouwels et al., 1996). The reporter gene *gusA* was subsequently deleted using *Xho*I restriction sites cutting followed by ligation and introduction into *E. coli* DH5 α by electroporation. The selection of positive clones was performed on LB-plates containing 100 μ g/ml of ampicillin. The scFv-E-tag fused to the long anchor was excised from the pLP402-scFv-long anchor construct by restriction cut at the *Cla*I and *Nhe*I sites and ligated into the pLP502 vector to generate an anchored product and then introduced into *E. coli*. After isolation of plasmids using the QIAprep[®] Spin Miniprep kit (QIAGEN, Hilden, Germany), *Tldh* was removed by *Not*I cutting. The electroporation of *E. coli* and plasmid isolation was then repeated as described above. One μ g of plasmid DNA and 100 μ l competent *L. casei* 393 pLZ15⁻ (recently reidentified as *L. paracasei*) (Perez-Martinez, 2003) were used for transformation by electroporation in the Gene Pulser II at 6.250 V/cm², 25 μ F, 100 Ω as described previously (Pouwels et al., 1996). Selection of positive clones was performed using MRS-plates containing 3 μ g/ml erythromycin. At each step of the construction process, the gene expression cassette was sequenced (Big Dye Terminator v.2, Applied Biosystems Foster City, CA).

IN VITRO EXPERIMENTS

Different experimental *in vitro* models were used for verification and visualization of binding, surface detection of scFv on the of bacterial cell and agglutination ability. They are summarized and presented in table 3.

Table 3.

Overview of *in vitro* experiments used in the study

Paper	Experimental model	References
II	ELISA, Western blot, Flow cytometry, SEM (scanning electron microscopy), agglutination assay	Carlsson et al., 1975, Laemli 1970, Towbin et al., 1979, Boyde and Stewart, 1963
III	Agglutination assay, inhibition assay	Schilling and Bowen, 1992
IV	ELISA, competitive ELISA, Western blot	Carlsson et al., 1975, Laemli 1970, Towbin et al., 1979,
V	ELISA, Western blot, SEM, growth inhibition assay, agglutination assay	Carlsson et al., 1975, Laemli 1970, Towbin et al., 1979, Boyde and Stewart, 1963

IN VIVO EXPERIMENTS

In the area of oral microbiology, laboratory animals have been used to study diseases of the oral cavity, of which dental caries has been studied extensively. Dental caries may be characterized as a localized, progressive, molecular disintegration of tooth structure. In the field of oral vaccines the use of animal models for evaluating the protective effects of antibody therapy is extremely valuable. Although, the anatomic structure of human and rodent teeth differs in certain respects, the appearance of a caries lesion in a human being is similar to that of a rat.

The desalivated rat model

The progression of dental caries is usually slow in humans as well as in rats. Bowen et al., have established a rat model where decrease of the salivary secretion will create a dry mouth situation in the oral cavity of rats (Bowen et al., 1988).

Sprague Dawley SPF rats are anesthetized intra-peritoneally with a combination of Hypnorm® and Dormicum® that will provide profound analgesia and good muscle relaxation. The parotid ducts are ligated by passing of a suture around the duct. The submandibular and sublingual salivary glands are removed following blunt dissection through a midline incision, and the wounds on the cheek and throat are closed by sutures (Bowen et al., 1988). After infection with *S. mutans*, both smooth and sulcal coronal caries will develop rapidly, sufficient disease is present within 21 days to permit evaluation of potential therapeutic agents. The pattern of the disease resembles the caries in dry mouth patients and was used in all rat experiment included in this thesis.

The Keyes index for evaluation of dental caries in rats

Already 1958, Paul Keyes established an index for evaluation of dental caries in rats and rodents. In this index various types of caries lesions found in the molar dentition of the rat can be evaluated by estimating the linear spread in one plane and by characterizing the depth of penetration. These features have been incorporated into a standardized system of scoring which is consistent and offers the advantage of describing and distinguishing differences in the distribution and extent of caries lesions (Keyes et al., 1958).

RESULTS

PAPER I

In this paper we summarized the most important work concerning passive immunotherapy using bovine immunoglobulins directed against a variety of microorganisms in the oral and gastro-intestinal tract, including *S. mutans* and *C. albicans*.

We also included original data from *in vitro* experiments. We found that polyclonal antibodies from cows immunized with whole *Candida albicans* organisms and purified mannan, inhibited binding of yeast cells to buccal epithelial cells in a dose-dependent manner.

PAPER II

Lactobacilli have previously been used to deliver vaccine components for active immunization *in vivo*. In this paper we describe a novel method where single-chain Fv (scFv) are produced *in situ* for delivery of passive immunity. Vectors encoding an scFv antibody fragment, which recognizes the streptococcal antigen I/II (SAI/II) adhesion molecule of *S. mutans*, were constructed and expressed in *Lactobacillus zeae*. The scFv antibody fragments, secreted into the supernatant or expressed on the surface of the bacteria, showed binding activity against SAI/II in enzyme-linked immunosorbent assay (ELISA). The scFv was detected on the surface of transformed *L. zeae* by Flow cytometry and binding of Lactobacilli expressing the scFv fragment fused to an E-tag were visualized by scanning electron microscopy (SEM), using beads coated with a monoclonal anti-E-tag antibody. Surface scFv-expressing lactobacilli agglutinated SAI/II-expressing *S. mutans* without affecting the corresponding SAI/II knockout strain. Oral administration of scFv-expressing bacteria in desalivated rats resulted in less *S. mutans* bacteria and lower development of dental caries.

PAPER III

Passive immunization using chicken anti-Gtf antibodies has previously been shown to protect rats against dental caries and reduce the number of *S. mutans* in humans. In this study we evaluated the anti-cariogenic effect of chicken anti-Gtf antibodies dissolved in

drinking water of desalivated rats. *In vitro*, the chicken anti-Gtf antibodies were able to reduce the activity of both GtfB and GtfC in solution, and when enzymes were coated on the surface of hydroxyapatite beads. Rats receiving chicken anti-Gtf antibodies in their drinking water had lower number of *S. mutans* and displayed a lower development of smooth and sulcal dental caries in comparison to rats given non-immunized egg yolk powder or sterile water. These results show a therapeutic effect of chicken derived antibodies in rats lacking salivary secretion, suggesting future potential therapy for patients with dry mouth syndrome suffering from high rate of caries development.

PAPER IV

Llama, dromedaries and camels produce IgG antibodies devoid of light chains and CH1 domains consisting of only the heavy chain part of the immunoglobulin molecule. They display high affinity and binding capacity and are more heat and acid resistant than conventional Ig. The variable domain is referred to as VHH, and two different llama VHHs, S36-VHH and S36-VHH-GOx, directed against *S. mutans* were constructed and produced in *Saccharomyces cerevisiae*. In S36-VHH-GOx the VHH was fused with glucose oxidase, which has previously been shown to express bactericidal activity *in vitro*. We found in Western blot analysis that the S36-VHH recognizes the SA I/II adhesion antigen of *S. mutans*. In competitive ELISA, the S36-VHH showed binding activity against the SA I/II surface antigen of *S. mutans* without competing with the monoclonal Guy's 13 antibody, suggesting binding to a different epitope than the mouse monoclonal. Rats receiving a daily oral dose of S36-VHH or S36-VHH-GOx had a lower development of smooth surface caries in comparison to irrelevant VHH and sterile water controls. We could not detect an additional protection of the glucose oxidase fusion protein, suggesting a low *in vivo* effect of GOx. These results show, for the first time, a functional therapeutic capacity of llama VHH.

PAPER V

In this paper we describe a further development of the first series of lactobacilli constructs presented in paper II. The previous constructs contained an inducible promoter region thereby dependent upon the presence of mannitol for expression of scFvs. In this set of new constructs the inducible promoter has been replaced and the new constructs have a continuous expression of antibody fragments directed against the

SA I/II antigen. The expression of scFv on the surface of *L. zaeae* showed binding to SA I/II, verified by ELISA and immunoblotting methods. The amount of surface expression of scFv was equal to the previous construct and showed binding to beads coated with SA I/II antigen visualized by SEM. Lactobacilli transformed with the 502-long anchor construct were able to agglutinate *S. mutans* to the same degree as the previous 402-long anchor construct. The protective effect against dental caries was evaluated in a desalivated rat model. We found that rats treated daily (swabbing and in drinking water) with *L. zaeae* 502-long anchor had a lower development of smooth surface caries in comparison to controls. The protection was slightly better to that found in the 402-long anchor construct group.

DISCUSSION AND FUTURE ASPECTS

Dental caries remains one of the most widespread diseases of mankind and continues to plague most of the world populations despite advances in prophylactic measures to deal with this disease (Bowen, 2002). In Sweden, the mean number of DFS (decayed and filled surfaces) of children has decreased from 1973 to 1993 (Hugosson et al., 2000). Interestingly, most of this decrease took place during the first five years between 1975-78, and in the age groups 15 and 20 years, the number of decayed surfaces (DS) increased after 1978 reaching the same level in 1993 as in 1973. Caries epidemiology is usually presented using mean values, which can be doubtful due to that data obtained from these studies are often not normally distributed (Bowen 2002). Clearly, the incidence of caries has been reduced in some segments of the population due to prophylactic measures during the past decades (Hugosson et al., 2000). However, despite oral hygiene instructions, fissure sealants and fluoride prophylaxis there exist groups that continue to suffer from dental caries and there are no signs of improvement. Also, patients suffering from hyposalivation, induced by certain pharmaceutical products or local radiation therapy due to tumors in the head and neck area, or patients with Sjögren's syndrome. These patients harbour a more caries related micro-flora (Almståhl and Wickström 1999) and develop caries rapidly (Spak et al., 1994). For these patient groups, an individual prophylaxis is important and due to the fragile oral mucosa following hypo salivation, a mouth rinse containing antibodies against *S. mutans*, may, in combination with fluoride, be a suitable treatment to decrease the cariogenic micro-flora and reduce the effects of demineralization. In our study, we clearly show that chicken anti-Gtf antibodies, when applied in drinking water, were functional and protective in a dry mouth environment in desalivated rats infected with *S. mutans* (Paper III).

Caries lesions result from the interaction of the bacteria, which colonize or infect the tooth surface with constituents of the diet, usually sucrose. A caries lesion is the clinical manifestation of a pathological process that may have been occurring on the tooth surface for months or years. The initial attachment to the saliva pellicle and later on, the survival in the acid environment, are virulence factors of great importance for the ability of *S. mutans* to colonize the tooth. Therefore, the enzymes and molecules involved in this process have been the focus as targets for vaccine development. The SAI/II antigen is suggested to be involved in the sucrose-independent attachment to the

tooth pellicle (Koga et al., 2002) and seems to be less important when sucrose is present in the environment. However, several studies both in animals and humans have reported a protective effect against dental caries in both active and passive immunization studies (Koga et al., 2002) with sugar present. Glucosyltransferases, on the other hand, are involved in the sucrose-dependant adherence and are important both for binding to the pellicle (Venkitaraman et al., 1995) and glucan, the extra cellular polysaccharadide produced, is an energy source in case of starvation. Lately, recombinant proteins of both these virulence factors have been evaluated using both active and passive immunization routes (Koga et al., 2002).

Active immunization has been successful in attaining an immunological response of both secretory and systemic nature. The previous problems with cross-reactive antibodies seem to be solved using purified parts of the antigen or recombinant protein or synthetic peptides (Hajishenghallis et al., 1998, Taubman et al., 2000, 2001). Still, there are potential risks involved when dealing with the systemic immune system. Active immunization as vaccination route, gastric or intranasal administration, is based on the idea that the same principles that apply to mucosal immunity, are applicable to protection against caries. Dental caries does not occur on a mucosal surface but on the hard tooth surface and protective antibodies should react on a solid surface in a hostile environment with large variations in pH values, active proteases, and limited difussion into and out of the plaque.

Furthermore, there is no evidence that resistance to caries is associated with elevated levels of antibodies to *S. mutans*. The correlation between salivary IgA and DMF index has been found to be positive, negative, or not significant (Marcotte and Lavoie, 1998). Similarly confusing data have been found when trying to correlate salivary IgA and *S. mutans* and active caries (Marcotte and Lavoie, 1998). If salivary IgA plays a major role in the preservation of the homeostasis of the oral micro-flora, IgA-deficient individuals should show an increased susceptibility to caries disease. The few studies performed give ambiguous results (Marcotte and Lavoie, 1998).

Passive immunization routes have succeeded in decreasing the number of *S. mutans* and lower the caries development in animal experiments (Koga et al., 2002) and in humans, local application of monoclonal antibodies is protective against dental caries (Ma et al., 1998). The sources of antibodies have been immunized cows (Michalek et al., 1987), hens (Hamada et al., 1990) or genetically modified tobacco plants (Ma et al., 1995). Longer-term effects on the indigenous micro-flora have been reported and

inhibition of re-colonization of *S. mutans* has been observed for at least 4 months. The method is local and safe, because there is no involvement of the systemic immune system and there are no side effects reported. Passive immunization should rather thus probably needs to be repeated in caries active patients several times per year. In contrast, the cariostatic effect of fluoride is heavily dependent on its ambient levels in the oral cavity and needs to be administered constantly (Corpron et al., 1986). Fluoride is not completely effective but can control demineralisation, but does not prevent the disease. Still, passive immunization is expensive and new traits need to be explored in order to find a safe and cost efficient therapy for oral diseases. Studies evaluating the effects of a caries vaccine should be compared to the effects of fluoride and a future strategy may be to combine them.

Lactobacilli are considered as GRAS organisms and their health promoting effects are well recognized. In the oral cavity, lactobacilli have historically been correlated to dental caries due to the presence in carious dentine and production of lactic acid. There is a large variation between different strains of lactobacilli regarding lactic acid production and secretion of inhibitory substances, such as bacteriocins, and we have not detected any additional cariogenic effect of the *L. casei* ATCC 393 used in our rat experiments. As delivery vehicles in the oral cavity, lactobacilli have several advantages. They are acid tolerant and can survive in the close vicinity (plaque) of *S. mutans* and their capacity of lactic acid production varies greatly between different strains, making it possible to choose a less acidogen strain, thereby solving the problem of prolonged pH drop and possible effect on the caries decay. Lactobacilli are part of our indigenous micro-flora and a natural ingredient of daily food without causing any immunological response. Previous studies in children have shown that *L. rhamnosus* GG when mixed with milk, was even able to decrease the number of *S. mutans* and development of dental caries (Näse et al., 2001).

In vitro, we found that when lactobacilli were cultured together with *S. mutans*, they had a strong growth inhibitory effect on *S. mutans* and the effect persisted although the strain was not able to produce lactic acid (Paper V). The inhibitory effect could be due to secretion of bacteriocins but this needs to be further investigated. Consequently, we may have a synergistic effect of our delivered antibody fragments. We have already sampled and characterized several human oral strains of lactobacilli and in the future we would like to choose a strain for transformation that possesses the best characteristics to

colonize the human oral cavity. Cultivation of *Lactobacillus* is inexpensive and high concentrations are easily achieved.

We have for the first time expressed on the surface of *L. zeae*, a functional scFv, in our case, directed against the SAI/II antigen of *S. mutans* (*L. casei*) (Paper II). The hypothesis was that after colonization of the transformed lactobacilli, the *in situ* delivery of scFv should be protective against dental caries. When administered to rats, the scFv expressing lactobacilli decreased the number of *S. mutans* and the development of dental caries (Paper II). The plasmid based system developed for expression of scFv directed against SAI/II of *S. mutans*, used in this study, is instabile and needs antibiotic resistance markers to force the bacteria to keep the plasmid. This system is therefore not suitable for human studies, but development of a chromosomal integrated system for expression of scFv is ongoing. The chromosomally integrated system is stabile and without antibiotic resistance markers. With the chromosomal system it is possible to enter numerous genes and express antibody fragments directed against several epitopes achieving synergistic effects. One suggestion is to include antibodies against both the SAI/II and Gtfs. For administration in humans several delivery routes can be optional. In dry mouth patients a rinsing solution could be preferable but in patients with normal saliva a tablet with slow release of lactobacilli expressing scFv could be suitable.

Llama, dromedaries and camels produce IgG antibodies devoid of light chains and CH1 domains and consist of only the heavy chain part of the immunoglobulin molecule (Hamers-Casterman et al., 1993). They display high affinity and binding capacity and are more heat and acid resistant than conventional Ig. The variable domain is referred to as VHH (Desmyter et al., 2001) and is comprised of a single immunoglobulin domain. When administered daily in the oral cavity of desalivated rats, VHHs directed against the SAI/II antigen of *S. mutans* were able to decrease the development of dental caries (Paper IV). We have for the first time shown, that llama VHH are functional *in vivo*, but further studies are required. These results are interesting since Ma et al., found that F(ab) fragments failed to reduced the colonization of *S. mutans*, but when applied to the teeth of healthy volunteers (Ma et al., 1990). This may be due to that Fab fragments may be more susceptible to proteolysis. Although, F(ab) fragments retain their full antigen-binding capacity and may block adhesion (Hajishengallis et al., 1992, Mansa et al., 1986), they are probably unable to aggregate or to reduce the hydrophobicity of bacteria. Our results point to the feasibility of using llama derived VHHs in passive immunization therapy for dental caries.

We have already constructed a lactobacilli transformant expressing the VHH directed against SAI/II on the bacterial surface. Expression of llama VHH on the surface of lactobacilli could be beneficial. The VHH fragment is smaller and can thereby easily fold correctly on the surface, and is also more acid resistant than the scFv, which of course is an advantage in the acid plaque environment. Further evaluation *in vivo* in the desalivated rat model is planned.

The mechanism of action of the anchored scFv or llama VHH needs to be further investigated. The desalivated rat model is highly cariogenic and the rats consume high concentrations of sucrose, which should theoretically rule out an importance of the SAI/II antigen according to previous studies (Koga et al., 2002). The anti-cariogenic effect seen in the desalivated rat model is probably due to the agglutination of *S. mutans* rather than blocking of initial binding of the SAI/II epitope. Our studies point to the possibility of an aggregation mediating effect of the SAI/II antigen since neither our transformed lactobacilli, or the wild type *L. zeae*, are able to aggregate the SAI/II knock out strain (Paper II, V), but this needs to be investigated further.

GENERAL SUMMARY AND CONCLUSIONS

Most of the diseases that affect the oral cavity are caused by the indigenous micro-flora when the delicate equilibrium has been altered within the niche. Passive immunization is a natural way of providing an immunological protection in the gastro-intestinal tract of young children when their immune system has not yet matured. Several previous investigations have shown the efficacy of antibodies, originating from immunized animals, when applied locally in order to decrease the development of dental caries. It is safe and the antibodies can be administered locally on the site of infection. Still, passive immunization is an expensive therapy and future research needs to focus on developing novel systems for production of antibodies.

The following conclusions can be drawn from the herein presented work:

- Bovine antibodies can prevent binding of *C. albicans* to human buccal epithelial cells thereby preventing disease.
- Chicken anti-Gtf antibodies have the ability to *in vitro* block enzymatic activity both in solution and on the surface of saliva coated hydroxyapatite beads.
- Chicken anti-Gtf antibodies applied in drinking water to desalivated rats were able to decrease the development of smooth and sulcal caries in comparison to non-immunized egg yolk control and sterile water control.
- A new system of surface expression of scFv directed against the SAI/II antigen of *S. mutans* thru transformation of *L. zae* using vector constructs has been established.
- The scFvs expressed anchored on the surface of lactobacilli show binding capacity to the SAI/II antigen, are detected on the surface of lactobacilli and aggregates *S. mutans* wild type strain *in vitro*.
- When the transformed lactobacilli were applied through oral swabbing and in the drinking water to desalivated rats, they were able to decrease the number of *S. mutans* and lower the development of smooth surface caries.
- Llama VHH fragments applied daily in the oral cavity of desalivated rats were able to decrease the development of total smooth surface caries.

- A new series of vector constructs with continuous expression of scFv have been developed and a functional scFv directed to SAI/II antigen has been expressed anchored on the surface of *L. zeae*.
- The continuous expression of scFv lowered the development of total smooth surface caries in desalivated rats and was slightly better when compared to the previous expression constructs employing an inducible promoter.

The aim of the present study was to develop a safe, cost efficient and local passive vaccine to prevent disease caused by oral pathogens. Dental caries is an infectious disease caused by *S. mutans*, a bacteria with specific abilities to colonize and survive on the tooth surface. We have developed, evaluated and demonstrated the anti-cariogenic effect of oral administration of antibodies or antibody fragments through drinking water, as a pipetted dose or delivered by transformed lactobacilli, in a desalivated rat model of dental caries. The latter is a new strategy for cost efficient production of *in situ* delivery of passive immunity. The antibodies or antibody fragments (scFv, VHH) applied, were directed against either Gtf or the SAI/II antigens of *S. mutans*, two of the most important virulence factors. Our performed studies clearly show that delivery of protection through passive immunization could be a suitable therapy against dental caries. Also, *in situ* delivery of scFv expressed on the surface of transformed lactobacilli is a new cost efficient method for passive immunization against dental caries.

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