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Short communication

Influence of microparticle formulation on immunogenicity of SYI, a synthetic peptide derived from *Streptococcus mutans* GbpB

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Peacock ZS, Barnes LA, King WF, Trantolo DJ, Wise DL, Taubman MA, Smith DJ. Influence of microparticle formulation on immunogenicity of SYI, a synthetic peptide derived from Streptococcus mutans *GbpB.*
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Subcutaneous immunization with SYI, a peptide construct based on *Streptococcus mutans* glucan binding protein B (GbpB) residues 113–132, significantly reduces experimental dental caries. Since mucosal immunization may be preferred for human vaccine applications, the present objective was to determine what formulation of SYI combined with polylactide-coglycolide microparticles could give rise to significant levels of salivary IgA antibody reactive with the native GbpB protein. A comparison of the SYI construct, loaded into or mixed with polylactide-coglycolide revealed the SYI-loaded microparticles to induce significant and sustainable levels of salivary and nasal wash IgA antibody to the peptide and the native protein. SYI mixed with unloaded microparticles was less effective in mucosal antibody response induction. These studies indicate that mucosal immunization with the SYI construct can induce salivary IgA antibody to a pathogenesis-associated component of *S. mutans* if delivered within polylactide-coglycolide microparticles, suggesting that this approach could successfully induce protective salivary immunity to dental caries caused by *S. mutans.*

Key words: dental caries; GbpB; IgA; polylactide-coglyclolide; *Streptococcus mutans*

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Glucan-mediated accumulation is strongly implicated in the molecular pathogenesis of dental caries associated with *Streptococcus mutans* (4). This accumulation may occur, in part, through the binding of glucan to one of several glucan binding proteins (1, 2, 10, 12) on the bacterial cell surface. One of these, glucan binding protein B (GbpB) is of particular interest because systemic (18) or mucosal (13) immunization with GbpB has been shown to induce protective immune responses against experimental dental caries and because children who become infected with *S. mutans*, often form salivary IgA antibody to this protein (14). We have recently cloned and sequenced GbpB (6), in part to identify domains which may be used in subunit vaccines. Since analysis of the primary sequence revealed no regions of obvious function that could be used to target immune responses, we applied a matrix-based algorithm for T-cell epitope prediction (8) to prospectively identify conserved class II-restricted major histocompatibility complex (MHC) ligands in the GbpB sequence. This approach revealed potential immunogenic peptide candidates, one (SYI) of which was synthesized, shown to be immunogenic, eliciting antibody that was reactive with the parent GbpB protein and able to induce dental caries-protective immunity after systemic immunization (16).

It has been suggested that mucosal immunization would be the preferred route for application of dental caries vaccines to humans since this approach could focus a majority of the immune response in the oral cavity, thereby maximizing levels of putatively protective salivary IgA antibody (3, 9). Little evidence exists for induction

of caries-protective responses by mucosal application of synthetic peptides derived from *S. mutans* glucosyltransferases (GTF) or glucan binding proteins. However, the SYI peptide construct has been shown to be the most immunogenic of all GbpB peptides evaluated thus far, when administered subcutaneously (11, 16). Furthermore, previous experiments with the *S. mutans* GTF peptide construct, HDS, revealed that this peptide, when incorporated into polylactide-coglycolide (PLGA) microparticles (5) and delivered intranasally with cholera toxin, would induce salivary IgA antipeptide antibody in most animals (15). Thus we hypothesized that intranasal application of the GbpB peptide, SYI, together with cholera toxin, would also be immunogenic. Furthermore, since the formulation of PLGA and peptide required to achieve a mucosal response in the presence of cholera toxin was not clear from our earlier work (5), we compared the mucosal and systemic antibody responses to SYI when peptide was loaded into, or simply mixed with PLGA.

The SYI peptide (KSNAATSYI-NAIINSKSVSD; GbpB residues 113–132) was synthesized (AnaSpec, Inc., San Jose, CA) as a multiple antigenic peptide (MAP) construct (20) on a core matrix of lysines to yield macromolecules with four peptides per molecule (7). In one formulation, the SYI peptide construct was administered as a mixture with unloaded PLGA microparticles (70% lactide, 30% coglycolide), to which 1% gelatin had been added (Up). In a separate formulation, SYI was incorporated into PLGA microparticle preparations to achieve 4% loading of peptide (Lp). Unloaded and loaded microparticles were prepared by Cambridge Scientific Inc. (Cambridge, MA) as previously described (19). GbpB was purified for ELISA from *S. mutans* strain SJ culture supernatants by ion exchange chromatography on MONO-Q HR 5/5 (Pharmacia, Piscataway, NJ) in the presence of urea (13).

Groups of 40-day-old Sprague-Dawley CD strain rats were immunized on days 0, 7 and 14 (primary). The first group ($n = 6$) was sham-immunized intranasally with unloaded microparticles (UN). A second group ($n = 6$) was immunized intranasally with PLGA microparticles loaded to 4% (60 μg) with SYI (LpN). A third group ($n = 6$) was immunized intranasally with unloaded PLGA mixed with 60 μg SYI (UpN). For all three groups, 1.5 mg of microparticles was delivered in a volume of 0.015 ml into each nostril. All intranasally-immunized groups also received a subsequent immunization on day 124 (secondary) with a dose and formulation identical to the previous doses. All intranasal doses were administered with 5 μg cholera toxin (List Biological Laboratories, Campbell, CA**)**. An additional group of three rats was injected subcutaneously in the salivary gland region on days 0 and 14 with the SYI construct (30 μg) incorporated into complete (first injection) or incomplete (second injection) Freund adjuvant.

Blood was collected under anesthesia on days 25, 35, 46, 123 and 133 for measurement of serum IgG and IgA antibody, as previously described (16). Sera from coagulated and centrifuged blood were stored at −20°C. Saliva for the measurement of salivary IgA antibody was collected on days 21, 35, 46, 123 and 133, clarified by centrifugation and stored at −70°C as previously described (16). Nasal washes for measurement of IgA antibody were collected on days 46 and 133 by rinsing each nostril three times with 0.015 ml phosphate-buffered saline (PBS), then stored at −70°C (16).

Serum IgG and IgA, salivary IgA and nasal IgA antibodies were measured by an enzyme-linked immunosorbent assay (ELISA). Antibody to *S. mutans* GbpB and the SYI peptide construct was assayed in polystyrene microtiter plates (Flow Laboratories) coated with 0.5 μg/ml of GbpB or 2.5 μg/ml SYI, respectively. Antibody to cholera toxin was assayed in plates coated overnight at 4°C with 0.2 ml of 2.5 μg/ml of monosialoganglioside GM 1 (Sigma Chemical Co., St. Louis, MO) in pH 9.6 coating buffer; then washed in 0.05% Tween 20 with 0.15 M sodium chloride and coated overnight at 4°C with 1 μg/ml cholera toxin (Sigma) in Tween 20 with PBS, pH 7.5. Antibody activity was measured by incubation at dilutions of 1 : 200 or 1 : 400 (serum–SYI or GbpB), 1 : 40000 (serum–cholera toxin), 1 : 4 (saliva) or 1 : 16 (nasal washes). Plates were then developed for IgG or IgA antibody as previously described (17). Reactivity was recorded as absorbance (A_{405nm}) in a micro plate reader (Biotek Instruments, Winooski, VT). Data were expressed as ELISA units (EU), which were calculated relative to the levels of appropriate reference sera or saliva from intranasally immunized Sprague-Dawley rats. Dilutions of sera producing an A_{405nm} of approximately 1.0 were considered to be 100 EU for serum IgG and IgA antibody measurements. Dilutions of saliva producing an A_{405nm} of approximately 0.8 were considered to be 100 EU for salivary or nasal wash IgA antibody. Antibody was considered detectable if the EU were greater than two standard deviations (SD) above the mean of the sham-immunized group.

To measure the relative level of IgA protein in saliva and nasal washes, plates were coated with mouse monoclonal reagent to rat α chain (1 : 500) (Zymed, South San Francisco, CA). After 2 h incubation with 1 : 200 saliva or 1 : 48 nasal wash dilutions, plates were developed with biotinylated mouse monoclonal reagent to rat α chain, followed by avidin-alkaline phosphatase (Cappel, West Chester, PA) and p-nitrophenylphosphate to reveal relative levels of IgA. Reactivity was recorded as absorbance (A_{405nm}). Data were expressed as IgA units, a relative indication of IgA concentration, by comparison with a precalibrated rat salivary IgA reagent. Salivary and nasal wash IgA antibody activities (EU) were then divided by IgA units to adjust for differences in IgA concentration among samples.

Figure 1 illustrates the levels of IgA antibody to native *S. mutans* GbpB protein, to the SYI peptide construct, and to cholera toxin in saliva taken 46 (primary) and 133 (secondary) days following initial intranasal immunization. Significantly elevated salivary IgA antibody to the SYI construct (center panel) was detected in the SYI-loaded (LpN) group after both primary ($P < 0.02$) and secondary ($P < 0.05$) immunization. Similarly elevated levels of salivary IgA antibody were observed in the subcutaneously immunized group after primary immunization. Salivary IgA antibody to the peptide construct was detected in only 2/6 rats given the mixed formulation, in contrast to 5/6 LpN-immunized rats.

Salivary IgA antibody reactivity to *S. mutans* GbpB is also shown in Fig. 1 (left panel). Again, loading the SYI construct into the PLGA microparticles (LpN) significantly ($P < 0.03$) enhanced the levels of IgA antibody reactive with the native GbpB protein, compared with the sham-immunized group on day 46 in the mixed group (UpN). Salivary antibody levels recovered to nearly primary antibody levels after a single boost on day 122. A less vigorous, but significant ($P < 0.03$), salivary IgA antibody response to GbpB was observed in the injected group (pSC) on day 46. This response eroded to an insignificant level by day 133. Mixing SYI with PLGA (UpN) did not induce a significant level of IgA antibody to GbpB at either day 46 or 133.

The patterns of nasal wash IgA antibody levels to *S. mutans* GbpB, the SYI peptide

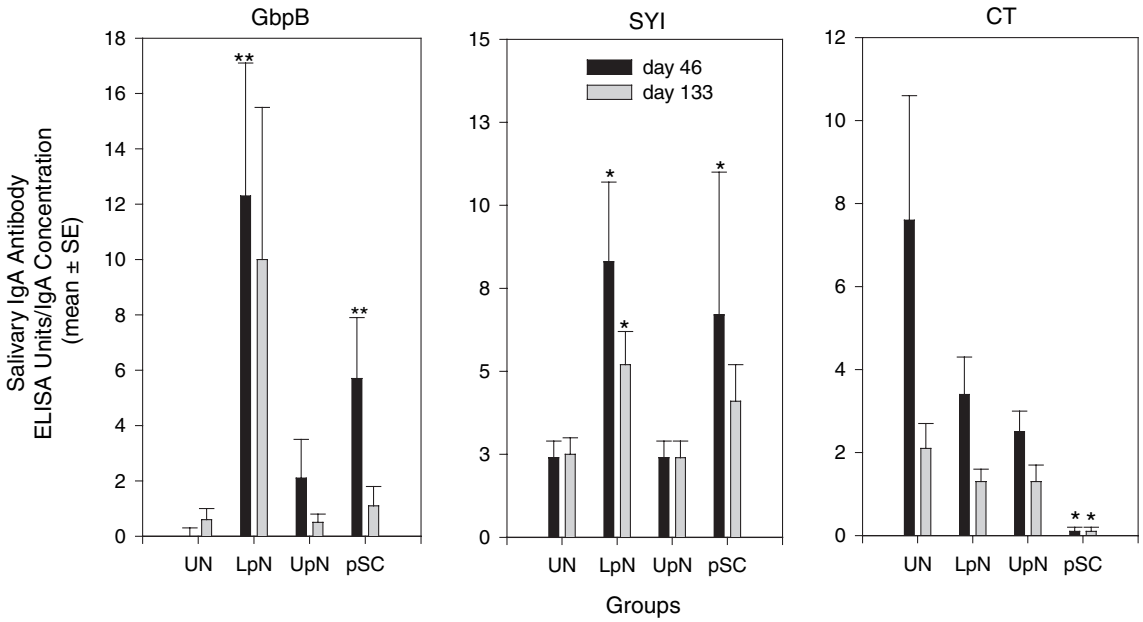

Fig. 1. Salivary IgA antibody activity (EU/IgA concentration) to *S. mutans* GbpB, the SYI MAP peptide construct, and cholera toxin (CT) 46 days (black bars) and 133 days (gray bars) after initial immunization. Groups were immunized intranasally with unloaded PLGA microparticles alone (UN), PLGA microparticles loaded with SYI peptide (LpN), or unloaded PLGA microparticles mixed with SYI peptide (UpN), or immunized subcutaneously with SYI peptide (pSC). Brackets enclose two standard errors. Asterisks in GbpB (left) and SYI (center) panels indicate significant differences (one-way ANOVA) between immunized and UN groups (**P* < 0.05, ***P* < 0.01), whereas in the CT panel (right) the asterisk indicates significant differences (**P* < 0.01) between the subcutaneously immunized (pSC) group and each group receiving cholera toxin as adjuvant (UN, LpN and UpN). Salivas were tested at 1 : 4 dilutions for IgA antibody levels and 1 : 200 for relative IgA protein levels.

construct, and cholera toxin following immunization with SYI (Fig. 2) are very similar to those observed in Fig. 1. The group immunized with SYI loaded into PLGA (LpN) had the highest mean IgA antibody levels to both the native GbpB protein (left panel) and the SYI construct (center panel) in nasal washes collected after primary or secondary immunization.

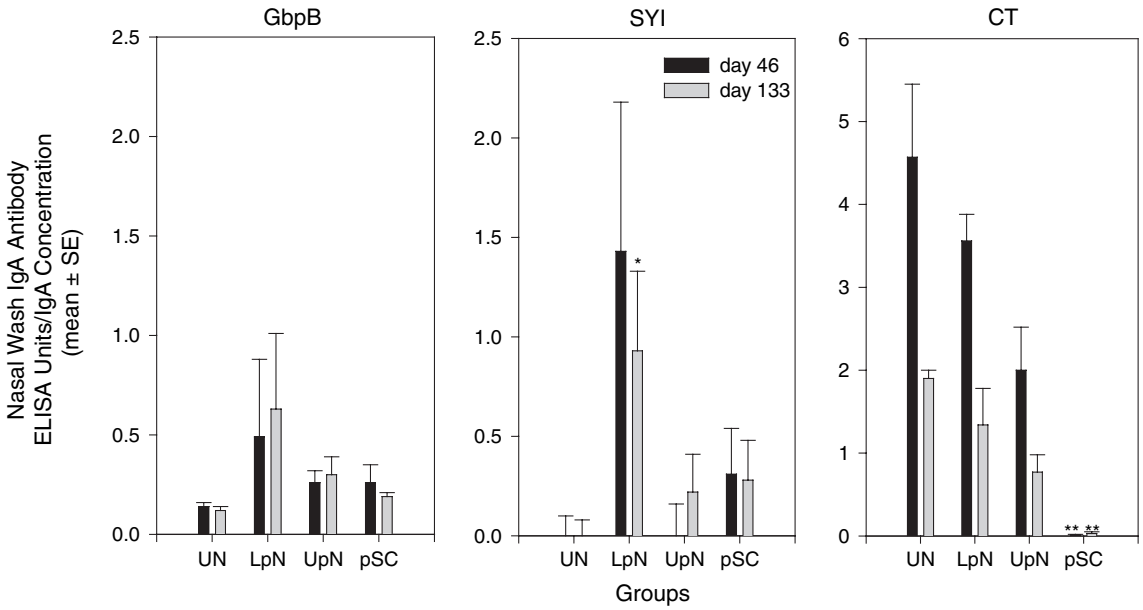

Fig. 2. Nasal IgA antibody activity (EU/IgA concentration) to *S. mutans* GbpB, the SYI MAP peptide construct, and cholera toxin (CT) 46 days (black bars) and 133 days (gray bars) after initial immunization. Groups were immunized intranasally with unloaded PLGA microparticles alone (UN), PLGA microparticles loaded with SYI peptide (LpN), or unloaded PLGA microparticles mixed with SYI peptide (UpN), or immunized subcutaneously with SYI peptide (pSC). Brackets enclose two standard errors. The asterisk in the SYI (center) panel indicates significant differences (one-way ANOVA) between immunized and UN groups (**P* < 0.05), whereas in the CT panel (right) the asterisks indicate significant differences (***P* < 0.01) between the subcutaneously immunized (pSC) group and each group receiving cholera toxin as adjuvant (UN, LpN and UpN). Washes were tested at a 1 : 16 dilution for IgA antibody level and 1 : 48 for relative IgA protein level.

At least four of six LpN-immunized rats had detectable levels of IgA antibody reactive with protein or peptide components. A lower frequency (1/6) and magnitude of response to these components was detected on days 46 and 133 in the rats immunized with SYI mixed with PLGA (UpN).

Salivary (Fig. 1, right panel) and nasal wash (Fig. 2, right panel) IgA antibody to cholera toxin was also measured. All groups receiving the cholera toxin responded with significant ($P < 0.01$) levels of salivary or nasal IgA antibody, compared to the subcutaneously immunized group on days 46 and 133.

Figure 3 presents the IgG antibody reactivity to *S. mutans* GbpB, to the SYI

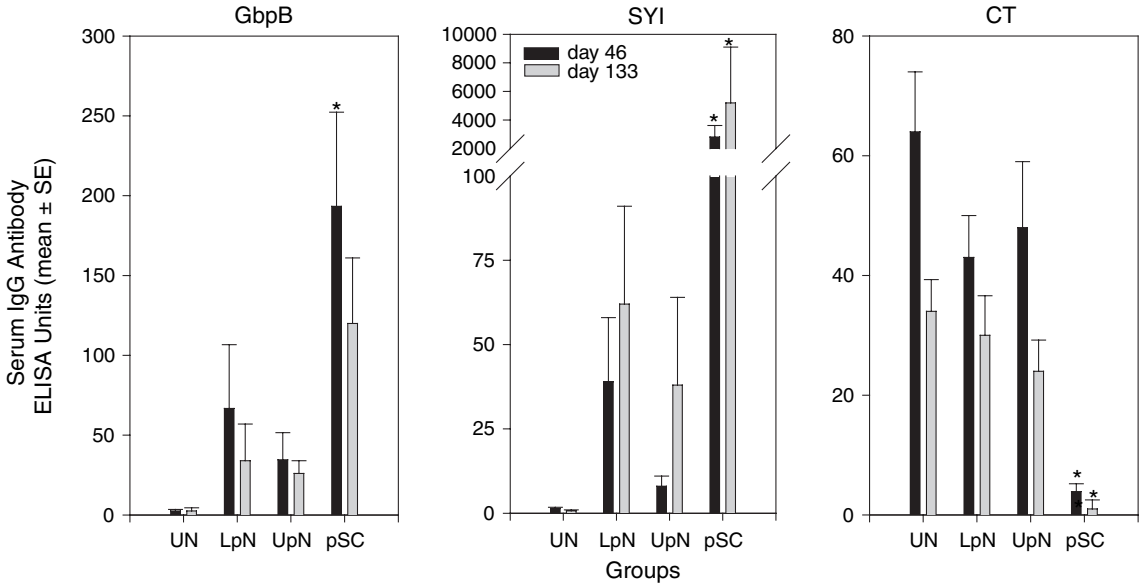

Fig. 3. Serum IgG antibody activity (EU) to *S. mutans* GbpB, the SYI MAP peptide construct, and cholera toxin (CT), 46 (black bars) and 133 days (gray bars) after initial immunization. Groups were immunized intranasally with unloaded PLGA microparticles alone (UN), PLGA microparticles loaded with SYI peptide (LpN), or unloaded PLGA microparticles mixed with SYI peptide (UpN), or immunized subcutaneously with SYI peptide (pSC). Brackets enclose two standard errors. Asterisks in GbpB (left) and SYI (center) panels indicate significant differences (one-way ANOVA) between immunized and sham-immunized (UN) groups (*$P < 0.05$), whereas in the CT panel (right) the asterisk indicates significant differences (*$P < 0.01$) between the subcutaneously immunized (pSC) group and each group receiving cholera toxin as adjuvant (UN, LpN and UpN).

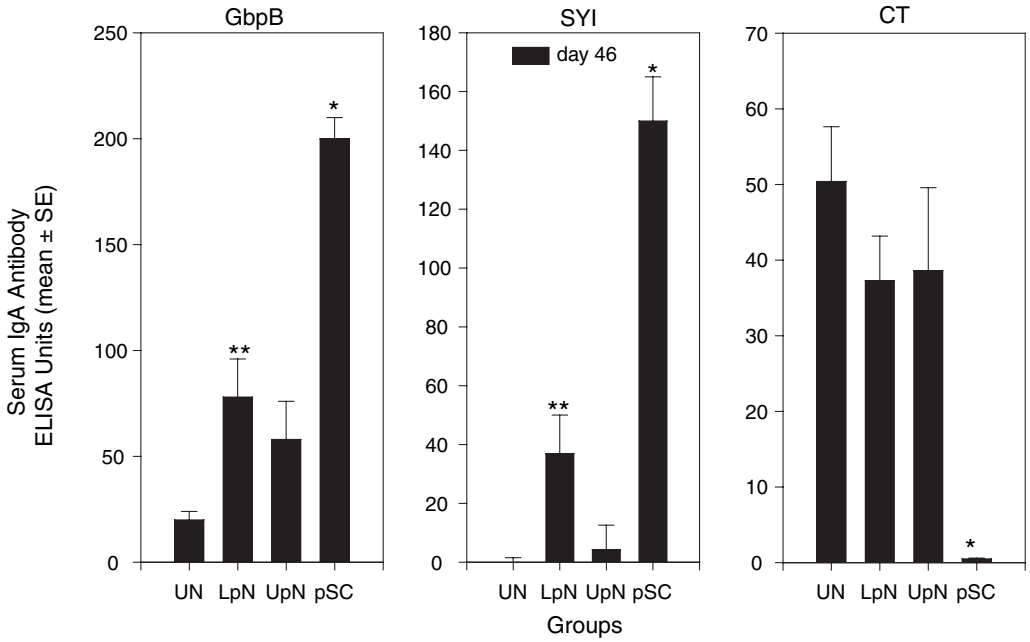

Fig. 4. Serum IgA antibody activity (EU) to *S. mutans* GbpB, the SYI MAP peptide construct, and to cholera toxin (CT) 46 days after initial immunization. Groups were immunized intranasally with unloaded PLGA microparticles alone (UN), PLGA microparticles loaded with SYI peptide (LpN), or unloaded PLGA microparticles mixed with SYI peptide (UpN), or immunized subcutaneously with SYI peptide (pSC). Brackets enclose two standard errors. Asterisks in GbpB (left) and SYI (center) panels indicate significant differences (one-way ANOVA) between immunized and UN groups (*$P < 0.05$, **$P < 0.01$), whereas in the CT panel (right) the asterisk indicates significant differences (*$P < 0.01$) between the subcutaneously immunized (pSC) group and each group receiving cholera toxin as adjuvant (UN, LpN and UpN).

peptide construct, and to cholera toxin in sera of immunized rats during the primary (day 46) and secondary (day 133) response to immunization with the SYI construct. Loading the SYI in the PLGA (LpN) resulted in a somewhat higher proportion of animals showing primary (4/6) and secondary (4/6) IgG antibody responses to SYI (Fig. 3, center panel), compared to animals immunized with the mixed (UpN) formulation (3/6 primary and 2/6 secondary). A similar relationship was observed between these two groups in their reactivity with the native GbpB protein (Fig. 3, left panel). Systemic immunization (pSC) induced markedly elevated ($P < 0.01$) serum IgG antibody levels to the SYI construct (center panel) and GbpB protein (left panel) on day 46. The serum IgG response to SYI remained at a high level on day 133. As seen with the mucosal IgA response, serum IgG antibody to cholera toxin (Fig. 3, right panel) was markedly elevated ($P < 0.01$) in all groups receiving the cholera toxin mucosal adjuvant.

Figure 4 presents the serum IgA antibody reactivity for the three components on day 46. Similar to the patterns observed after serum IgG antibody measurement (Fig. 3), the LpN group serum IgA antibody levels to SYI and GbpB were elevated, in both cases reaching significance ($P < 0.05$). Again, all groups exposed to cholera toxin had significantly elevated ($P < 0.01$) levels of serum IgA antibody to this adjuvant.

Thus, mucosal IgA antibody to the pathogenesis-associated GbpB could be induced in saliva and nasal secretions after intranasal delivery of the SYI construct. Furthermore, physical loading of the SYI peptide into PLGA microparticles gave a superior mucosal immune response compared with simple mixing of peptide with unloaded microparticles. Enhanced responses may have occurred from increased nasal exposure of peptide via retention, and possible entry, of loaded microparticles into nasal mucosal epithelium. Such retention or entry should increase the likelihood of peptide uptake by resident antigen processing cells. Uptake at other inductive sites in the upper respiratory or oral regions also cannot be discounted. Furthermore, additional doses of the intranasally administered SYI-loaded microparticles sustained the levels of mucosal IgA antibody to the native protein in most animals that exhibited a primary response. These patterns of response were reflected to a lower degree in measures of serum IgG and IgA antibody.

Previous studies (11, 16) indicated that the SYI construct derived from *S. mutans* GbpB was exceptional among putative immunogenic GbpB peptides with regard to its ability to induce protective immunity after subcutaneous injection in the salivary gland region. Since GbpB is associated with the bacterial cell surface (6), elevated levels of salivary IgA antibody to an available SYI epitope could significantly diminish the colonization potential of *S. mutans* through simple bacterial aggregation. Antibody may also interfere with GbpB activity, although the functional domains of this protein have not yet been identified. This study establishes that mucosal (intranasal) immunization with the SYI construct can successfully induce salivary antibody, which can be sustained by boosting if administered in a formulation in which the peptide is loaded within PLGA microparticles. Such an approach could be expected to result in protective immunity to experimental dental caries, thus expanding the epitopes and strategies which might be employed in future dental caries vaccine applications.

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References

1. Banas JA, Russell RR, Ferretti JJ. Sequence analysis of the gene for the glucan-binding protein of *Streptococcus mutans* Ingbritt. Infect Immun 1990: **58**: 667–673.
2. Banas JA, Vickerman MM. Glucan-binding proteins of the oral streptococci. Crit Rev Oral Biol Med 2003: **14**: 89–99.
3. Childers NK, Tong G, Li F, Dasanayake AP, Kirk K, Michalek SM. Humans immunized with *Streptococcus mutans* antigens by mucosal routes. J Dent Res 2002: **81**: 48–52.
4. Hamada S, Slade HD. Biology, immunology and cariogenicity of *Streptococcus mutans*. Microbiol Rev 1980: **44**: 331–384.
5. Hsu YY, Gesser JD, Stewart RR, Trantolo DJ, Lyons CM, Simons GA, et al. Mechanisms of isoniazid release from poly (d,l-lactide-co-glycolide) matrices prepared by dry-mixing and low density polymeric foam methods. J Pharm Sci 1996: **85**: 706–713.
6. Mattos-Graner RO, Jin S, King WF, Chen T, Smith DJ, Duncan MJ. Cloning of the *Streptococcus mutans* gene encoding glucan binding protein B and analysis of genetic diversity and protein production in clinical isolates. Infect Immun 2001: **69**: 6931–6941.
7. Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc 1963: **85**: 2149–2154.
8. Roberts CGP, Meister GE, Jesdale SM, Lieberman J, Berzofsky JA, DeGroot AS. Prediction of HIV peptide epitopes by a novel algorithm. AIDS Res Hum Retrovir 1996: **12**: 593–610.
9. Russell MW, Childers NK, Michalek SM, Smith DJ, Taubman MA. A caries vaccine? The state of the science of immunization against dental caries. Caries Res 2004: **38**: 230–236.
10. Sato Y, Yamamoto Y, Kizaki H. Cloning and sequence analysis of the *gbpC* gene encoding a novel glucan-binding protein of *Streptococcus mutans*. Infect Immun 1997: **65**: 668–675.
11. Shen B, King WF, Taubman MA, Smith DJ. Immune potential of glutamine-rich peptides from *Streptococcus mutans* GbpB. J Dent Res 2004: **83** Special Issue A: Abstract 3705.
12. Smith DJ, Akita H, King WF, Taubman MA. Purification and antigenicity of a novel glucan binding protein of *S. mutans*. Infect Immun 1994: **62**: 2545–2552.
13. Smith DJ, Heschel RL, Melvin J, King WF, Periera MBB, Taubman MA. *Streptococcus mutans* glucan binding proteins as dental caries vaccines. In: Husband AJ, Beagley KW, Clancy RL, Collins AM, Cripps AW, Emery DL, eds. Mucosal solutions: advances in mucosal immunology, **Vol. 2**. Sydney: University of Sydney Press, 1997: 367–377.
14. Smith DJ, King WF, Akita H, Taubman MA. Association of salivary immunoglobulin A antibody and initial mutans streptococcal infection. Oral Microbiol Immunol 1998: **13**: 278–285.
15. Smith DJ, King WF, Barnes LA, Trantolo D, Wise DL, Taubman MA. Facilitated intranasal induction of mucosal and systemic immunity to mutans streptococcal glucosyltransferase peptide vaccines. Infect Immun 2001: **69**: 4767–4773.
16. Smith DJ, King WF, Barnes LA, Peacock Z, Taubman MA. Immunogenicity and protective immunity induced by synthetic peptides associated with putative immunodominant regions of *Streptococcus mutans* glucan binding protein B. Infect Immun 2003: **71**: 1179–1184.
17. Smith DJ, Lam A, Barnes LA, King WF, Peacock Z, Wise DL, et al. Remote glucosyltransferase-microparticle vaccine delivery induces protective immunity in the oral cavity. Oral Microbiol Immunol 2003: **18**: 240–248.
18. Smith DJ, Taubman MA. Experimental immunization of rats with a *Streptococcus mutans* 59-kilodalton glucan-binding protein protects against dental caries. Infect Immun 1996: **64**: 3069–3073.
19. Smith DJ, Trantolo DJ, King WF, Gusek EJ, Fackler PH, Gresser JD, et al. Induction of secretory immunity with bioadhesive poly (D,L-lactide-co-glycolide) microparticles containing *Streptococcus sobrinus* glucosyltransferase. Oral Microbiol Immunol 2000: **15**: 124–130.
20. Tam JP. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc Natl Acad Sci U S A 1988: **85**: 5409–5413.