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Immunogenicity and *In Vitro* and *In Vivo* Protective Effects of Antibodies Targeting a Recombinant Form of the *Streptococcus mutans* P1 Surface Protein

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Streptococcus mutans **is a major etiologic agent of dental caries, a prevalent worldwide infectious disease and a serious public health concern. The surface-localized *S. mutans* P1 adhesin contributes to tooth colonization and caries formation. P1 is a large (185-kDa) and complex multidomain protein considered a promising target antigen for anticaries vaccines. Previous observations showed that a recombinant P1 fragment ($P1_{39–512}$), produced in *Bacillus subtilis* and encompassing a functional domain, induces antibodies that recognize the native protein and interfere with *S. mutans* adhesion *in vitro*. In the present study, we further investigated the immunological features of $P1_{39–512}$ in combination with the following different adjuvants after parenteral administration to mice: alum, a derivative of the heat-labile toxin (LT), and the phase 1 flagellin of *S.* Typhimurium LT2 (FliCi). Our results demonstrated that recombinant $P1_{39–512}$ preserves relevant conformational epitopes as well as salivary agglutinin (SAG)-binding activity. Coadministration of adjuvants enhanced anti-P1 serum antibody responses and affected both epitope specificity and immunoglobulin subclass switching. Importantly, $P1_{39–512}$-specific antibodies raised in mice immunized with adjuvants showed significantly increased inhibition of *S. mutans* adhesion to SAG, with less of an effect on SAG-mediated bacterial aggregation, an innate defense mechanism. Oral colonization of mice by *S. mutans* was impaired in the presence of anti-$P1_{39–512}$ antibodies, particularly those raised in combination with adjuvants. In conclusion, our results confirm the utility of $P1_{39–512}$ as a potential candidate for the development of anticaries vaccines and as a tool for functional studies of *S. mutans* P1.**

Streptococcus mutans is a Gram-positive bacterium and an established etiological agent of human dental caries, a transmissible, chronic, nonlethal infectious disease with a worldwide distribution (1, 2). Adherence of *S. mutans* to the tooth surface involves two stages: a sucrose-independent stage and a sucrose-dependent stage (1, 3). The initial sucrose-independent step is mediated by a reversible interaction between a large (185-kDa) bacterial surface protein, P1 (also referred to as antigen I/II [Ag I/II], antigen B, or PAc), and a high-molecular-weight salivary glycoprotein, called gp340, adsorbed to the tooth enamel (4, 5). Ag I/II family molecules are present on virtually all oral streptococci and have also been identified in other *Streptococcus* species (6–8). Based on its primary sequence, P1 demonstrates several distinct features: a secretion signal sequence (amino acids [aa] 1 to 38); the N-terminal pre-A region (aa 39 to 185); a series of three alanine-rich tandem repeats called the A region (aa 186 to 464); a variable region, or V region, where strain-strain differences are clustered (aa 679 to 823); a series of three tandem proline-rich repeats, or the P region (aa 840 to 963); and C-terminal anchoring and *trans*-membrane sequences including an LPXTG sortase motif (aa 1486 to 1561) (9–12). Monoclonal antibodies (MAbs) have been used to identify functional segments within P1 (9, 13, 14). The crystal structure of much of the protein has been resolved, revealing a unique architecture. The A region forms an α-helix that intertwines with the helical P region to form a highly elongated 50-nm fibrillar stalk with a globular domain that includes the V region at the tip and three globular DE (immunoglobulin motif) variant IgG-like domains encompassing the carboxy terminus at the base (15, 16). The N terminus of the protein has not yet been crystallized but is known to interact with the C terminus and to contribute to the overall folding, stability, and function of the full-length molecule (17). Two adhesive domains have been identified and localized in the modeled structure near or within the globular regions located at the ends of the helical stalk (15, 16).

The P1 protein of *S. mutans* interacts in different ways with soluble, and tooth attached, forms of human salivary agglutinin (SAG), a multimeric protein complex (18). The binding of bacteria to either soluble or immobilized SAG determines whether the bacteria will be aggregated and ingested or will remain in the oral cavity and adhere to the tooth surface. When immobilized, SAG serves as a substrate for the adherence of *S. mutans* and subsequent biofilm formation leading to the onset of the tooth decay process (19, 20). In contrast, aggregation by fluid-phase SAG represents an innate host defense mechanism (18).

Since P1 contributes to the cariogenicity of *S. mutans*, antibodies against P1 functional domains can block bacterial adherence, thereby disrupting colonization of the oral cavity (21–23). Indeed, a recombinant P1 fragment, corresponding to the A region or the saliva-binding region (SBR), expressed in *Escherichia coli* has been successfully used, after genetic fusion with cholera toxin, to induce

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antibodies and T cells with potential protective effects against dental caries under experimental conditions (23, 24). Recently, we have shown that another P1-derived fragment generated in *Bacillus subtilis*, encompassing amino acids 39 to 512 and named $P1_{39–512}$, is immunogenic in mice and induces antibodies that interfere with *S. mutans* adhesive properties *in vitro* (25). We furthermore demonstrated that a mucosal delivery system based on genetically modified *B. subtilis* spores that express $P1_{39–512}$ *in vivo* induced specific antibodies in serum and saliva that interfered with *S. mutans* adhesion to abiotic surfaces without preventing bacterial aggregation (26). These findings highlight the need for an understanding of the immunological, structural, and functional characteristics of $P1_{39–512}$ as an alternative to the full-length protein as a target antigen to generate protective immunity against dental caries. In addition, the observation that systemic IgG enters the oral cavity via the gingival crevice and confers protection to dental caries suggests that parenteral routes should also be tested for potential anticaries vaccines (21, 27–29).

Adjuvants are present in most vaccine formulations, particularly those containing purified proteins, also named acellular vaccines. Aluminum salts (alum) are added as adjuvants in most presently used vaccines (30). However, several alternative compounds, including molecules of microbial origin, have received growing interest as potential vaccine adjuvants, such as derivatives of heat-labile toxin (LT) produced by some enterotoxigenic *Escherichia coli* strains (31) and flagellin (FliC) of *Salmonella enterica* serovar Typhimurium, a Toll-like receptor 5 (TLR5) agonist capable of triggering the innate immune system (32).

In the present study, we evaluated the immunological features and potential protective effects of $P1_{39–512}$ produced in *B. subtilis* strains. The recombinant protein allowed us to define further the epitope specificity of several different P1-specific MAbs known to interfere with the adhesive functions of *S. mutans*. In addition, parenteral coadministration of $P1_{39–512}$ and different adjuvants (alum, flagellin, or an LT derivative) permitted us to characterize the serum antibody responses with regard to specificity and subclass distribution as well as potential anticaries protective effects, including adherence inhibition *in vitro* and bacterial colonization *in vivo*. Our results demonstrate that $P1_{39–512}$ expressed in *B. subtilis* represents a promising antigen for the development of anticaries vaccines and a useful reagent for functional, immunological, and structural studies of the *S. mutans* P1 protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. mutans* strains (UA159, NG8, or P1-deficient mutant strain PC3370) were grown in Todd-Hewitt broth or in brain heart infusion (BHI) broth, each supplemented with 0.3% yeast extract, at 37°C in 5% CO_2 (5, 33, 34). *E. coli* (CG14) and *B. subtilis* (1012 or LDV701) strains were grown aerobically at 37°C with constant shaking in Luria-Bertani (LB) broth (29, 35). Cultures were supplemented with antibiotics as needed.

Purification of P1-derived fragments and protein adjuvants. Expression and purification of full-length P1 (CG14), truncated P1 fragments ($P1_{39–512}$) (NR21, LT1, and MA41), and the adjuvant proteins (LTK4R and FliC) in *E. coli* or *B. subtilis* strains were performed according to previously described protocols (36–38). The LT derivative LTK4R shows reduced toxicity compared to the reference LT form (38), while recombinant FliC represents the phase 1 flagellin type (named FliCi) originally expressed by *S.* Typhimurium LT2 (39).

P1-specific monoclonal antibodies. P1-specific MAbs (3-8D, 1-6F, 4-9D, and 4-10A) were obtained from hybridomas previously generated at the University of Florida (40). The binding characteristics and functional activity of these antibodies were reported previously (41–43).

Reactivity of anti-P1 MAbs with $P1_{39–512}$ determined by ELISA. Enzyme-linked immunosorbent assay (ELISA) plates were coated with 200 ng/well of recombinant soluble or heat-denatured (100°C for 10 min) $P1_{39–512}$ in carbonate-bicarbonate buffer (pH 9.6), and the reactions were performed with MAbs 3-8D, 1-6F, 4-9D, and 4-10A. Binding of MAbs to the immobilized antigen was detected by using peroxidase-conjugated anti-mouse IgG followed by development with 0.1 M *O*-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich) and 0.012% H_2O_2 diluted in phosphate citrate buffer (0.01 M; pH 5.0). The reactions were stopped with 2 M H_2SO_4, and the absorbance at 492 nm was measured in an ELISA plate reader (Multiskan EX; ThermoLabsystems, Finland). All samples were assayed in duplicate, and dilution curves were prepared for each sample.

Western blot analysis of $P1_{39–512}$ with anti-P1 MAbs. Recombinant $P1_{39–512}$ purified from *B. subtilis* was subjected to electrophoresis on a polyacrylamide gel (7.5%) and transferred onto nitrocellulose membranes. The membranes were blocked with phosphate-buffered saline (PBS)–Tween 20 (0.3%) and then incubated with anti-P1 MAb ascites fluids at a dilution of 1/3,000. The membranes were washed and incubated with an anti-mouse IgG conjugate labeled with peroxidase (Southern Biotechnology Associates) (1/3,000), followed by development with a solution of 4-chloro-1 naphthol. A positive-control reaction was carried out with control serum raised in mice immunized with anti-$P1_{39–512}$ and Freund's adjuvant (25).

Interaction between $P1_{39–512}$ and immobilized SAG and inhibition of *S. mutans* adherence to SAG by anti-$P1_{39–512}$ antibodies determined by surface plasmon resonance. Interaction of $P1_{39–512}$ with and inhibition of adherence of *S. mutans* NG8 to immobilized SAG were determined by surface plasmon resonance (SPR) using a BIAcore model 3000 instrument (GE Healthcare, Salt Lake City, UT, USA). Human SAG was prepared as described previously and immobilized on CM5 chips (GE Healthcare) (~1,500 resonance units [RU]), according to previously described methods (42). For protein interaction experiments, aliquots (10 μl) containing 500 ng of $P1_{39–512}$ were injected onto the chip surface and subsequently eluted with regeneration buffer (PBS-Tween [0.3%] with EDTA [10 mM], NaCl [100 mM], and NaOH [100 mM]) at the end of each assay. The signal from the control surface (FC1) (not coated with SAG) was subtracted from that of the test surface (FC2) (coated with SAG) to produce a sensorgram (change in resonance units [ΔRU]). For inhibition of adherence of *S. mutans* NG8, serum samples (pools) from the different immunization groups were used at a dilution of 1/100. The ΔRU was used to calculate the percentage of inhibition of adhesion {adhesion inhibition = 100 − [(ΔRU of *S. mutans* NG8 incubated with test serum × 100)/ΔRU of *S. mutans* NG8]}. Sera from sham-treated mice were used as a negative control, and values for these sera were subtracted from the final absorbance value as the background. Serum samples collected after the third dose were used in the assays. The final values represent results of three independent experiments. Data were analyzed by using BIAE valuation software (v4.2; BIAcore, University of Florida, USA).

Immunization regimen and sample collection. All experiments involving animals were performed with prior approval from the Committee on the Ethical Use of Laboratory Animals of the Institute of Biomedical Sciences of São Paulo University (protocol no. 013) and in accordance with guidelines for the care and use of laboratory animals of the National Committee on the Ethics of Research (CONEP). Groups of five female BALB/c mice, aged 6 to 8 weeks, were immunized with three subcutaneous (s.c.) doses given at intervals of 15 days. Each dose consisted of 10 μg of $P1_{39–512}$ protein (per animal) admixed with 12.5 μg of aluminum hydroxide (alum), 1 μg of LTK4R, or 5 μg of FliC. Control groups were sham immunized with PBS. Mice were bled by submandibular puncture on days 0, 14, 29, 44, 60, and 112. Samples were held at room temperature for 30 min and then incubated for another 30 min at 4°C. Following centrifugation (6,000 × *g* for 30 min at 4°C), sera were stored in aliquots at −20°C until use.

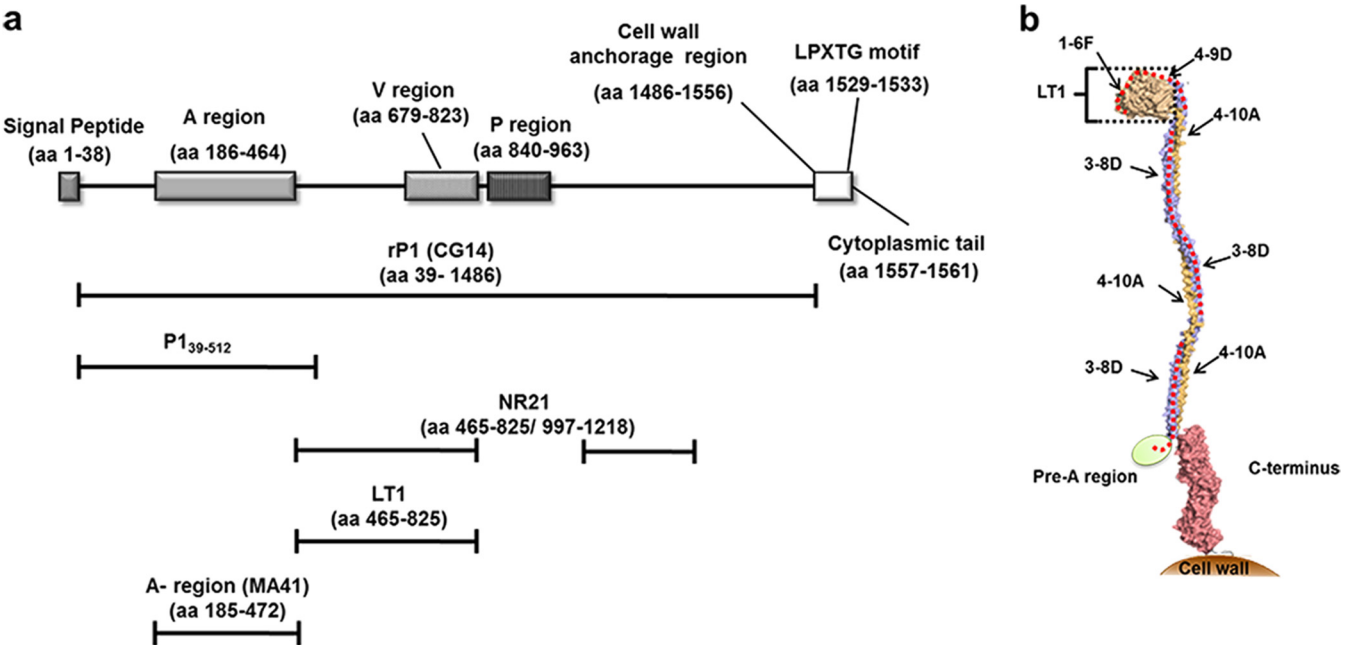

FIG 1 Schematic representation of functional and structural domains of the *S. mutans* P1 protein. (a) Linear representation of the protein sequence with the indicated functional/structural domains. The corresponding amino acid positions are shown in parentheses. Bars placed below the schematic indicate the recombinant proteins used in the present study: CG14 (recombinant full-length P1 sequence [rP1]), $P1_{39–512}$, MA41 (A region), LT1 (globular segment intervening between the A and P regions), and NR21 (fusion polypeptide lacking the P region). (b) Tertiary structural model of the *S. mutans* P1 protein. The location of $P1_{39–512}$ is marked by a red dotted line. The location of the LT1 polypeptide is indicated by a bracket. The approximate positions of experimentally determined epitopes recognized by the P1-specific MAbs (1-6F, 4-9D, 4-10A, and 3-8D) are indicated by arrows. The crystal structure of the N terminus has not yet been determined and is represented as an ellipse. It is known to interact with the C terminus to form a stable complex.

Determination of anti-P1 IgG responses, antibody avidity, and reactivity of anti-$P1_{39–512}$ antibodies with P1-derived fragments by ELISA. Serum samples were evaluated for their level of anti-P1 or anti-$P1_{39–512}$ total IgG or subclass titers (IgG1, IgG2a, and IgG2b), avidity, and recognition of P1 fragments by ELISA. To measure IgG responses, plates were coated with recombinant full-length P1 (CG14) or $P1_{39–512}$ (200 ng/well), and the assays were performed as previously described (25). The absorbance values obtained for samples collected from sham-treated mice were subtracted as the background. Serial dilution curves were drawn for each sample, and the endpoint titers were calculated as the reciprocal values of the last dilution with an optical density of 0.05. The avidity of serum antibody binding to $P1_{39–512}$ was determined by elution of ammonium thiocyanate, as previously described (44). After incubation with primary antibodies (all serum samples were normalized to an optical density at 492 nm [OD_{492}] of 1.0), plates were washed, and increasing concentrations of ammonium thiocyanate (0 to 8 M) diluted in PBS were added to the wells and incubated for 15 min. The concentration of ammonium thiocyanate required to dissociate 50% of the bound antibodies was determined. Percent binding of antibodies was determined by the use of the following formula: (OD_{492} in the presence of ammonium thiocyanate × 100)/OD_{492} in the absence of ammonium thiocyanate. The assay was performed with sera collected after the third vaccine dose. All samples were assayed in duplicate, and the experiments were performed independently at least twice with pooled sera from different immunization groups. To assess antibody specificity, the reactivity of sera from mice immunized with anti-$P1_{39–512}$ with and without adjuvant against truncated recombinant P1 fragments ($P1_{39–512}$, MA41, LT1, and NR21, as described above) was measured by ELISA (Fig. 1a).

Evaluation of antibody binding to P1 present on the surface of *S. mutans* by fluorescence-activated cell sorting (FACS). Binding assays were performed according to a previously described protocol (45). Briefly, *S. mutans* UA159 cells were grown in Todd-Hewitt broth supplemented with 0.3% yeast extract (THYE) to an OD_{600} of 0.5, washed once, and suspended in an equal volume of PBS. Bacteria were incubated for 30 min at 37°C with serum pools from each group (third dose) diluted 1/100. After additional washing, the bacteria were incubated with 100 μl of anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (MP Biomedicals) diluted 1:2,000. Finally, the samples were analyzed by using a FACScalibur instrument (BD Biosciences). Sera from sham-treated mice were used as negative controls, and only sera from the third dose of the different immunization groups were evaluated. All samples were assayed in duplicate, and the experiments were performed independently at least twice. Ten thousand gated events were acquired, and the median bacterial fluorescence intensity is shown for each sample.

Assessment of inhibition of SAG-mediated *S. mutans* aggregation. Assessment of the inhibition of SAG-mediated aggregation of *S. mutans* NG8 was performed according to a previously described protocol (46). The serum pools were tested at a final dilution of 1/100. The change in absorbance ($\Delta Abs_{600\ nm}$) was used to calculate the percentage of inhibition of aggregation {aggregation inhibition = 100 − [($\Delta Abs_{600\ nm}$ of *S. mutans* NG8 incubated with test serum × 100)/*S. mutans* NG8 $\Delta Abs_{600\ nm}$)]}. Values for samples from sham-treated mice were used as background controls, and values were subtracted from the final absorbance values for samples collected from the different immunization groups. Serum samples collected after the third dose were used in the assays. The final values represent results of three independent experiments performed in duplicate.

Serum neutralization of oral colonization of mice by *S. mutans*. The antiadhesive function of anti-$P1_{39–512}$ antibodies under *in vivo* conditions was determined by using a serum neutralization model developed in our laboratory. Briefly, 1 day before inoculation of bacteria, BALB/c mice (n = 5), aged 6 to 8 weeks, were given water containing 1% sucrose *ad libitum*, as described previously (47). The next day, the mice were anesthetized with ketamine (80 mg/kg of body weight) and xylazine hydrochloride (8

mg/kg of body weight), and the oral cavity of each animal was cleaned with chlorhexidine (0.12%) by using a sterile swab. Samples of endogenous oral microbiota were collected and plated onto LB and Mitis-Salivarius (MS) agar before and after treatment. After the oral cavity was washed with 1× PBS (pH 7.0), an aliquot (20 μl) of clarified saliva, prepared as described previously (48), was applied onto the tooth surfaces of each mouse by using a micropipette tip, and the animal was kept immobile for 30 s. Next, excess saliva was removed with a swab; immediately afterwards, an aliquot (20 μl) containing 3×10^9 CFU of *S. mutans* strain NG8 was applied to each mouse with a micropipette tip, and the animal was again kept immobile for 30 s. Bacteria were prewashed with TBSC (10 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$ [pH 7.6]) and suspended in TBSC for application. All procedures were performed while the animals were anesthetized. Animals remained anesthetized for 90 min. Oral colonization with *S. mutans* was determined 6 h after the mice returned from anesthesia by brushing the tooth surfaces with a sterile swab, resuspending the recovered bacteria in 1 ml of 1× PBS (pH 7.0), and plating samples onto MS agar plates containing bacitracin (0.2 U/ml) and sucrose (5%). Plates were incubated for 48 h at 37°C under anaerobic conditions, at which time CFU were counted. To evaluate antibody-dependent neutralization, prior to administration, bacteria were incubated with pooled sera from the various immunization groups for 30 min at 37°C. Pooled sera were diluted 1/100 in TBSC. Positive and negative controls included untreated *S. mutans* strain NG8 and the *spaP*-negative derivative PC3370, which is devoid of P1 (5), respectively.

Statistical analyses. The results were analyzed with the GraphPad Prism 5 program and were expressed as means ± standard errors of the means (SEM). Statistically significant differences were determined by two-way analysis of variance (ANOVA) and Bonferroni's multiple-comparison posttests.

RESULTS

Epitopes recognized by P1-specific antiadhesive MAbs are preserved within $P1_{39–512}$. Recombinant $P1_{39–512}$ expressed in *B. subtilis* strain LDV701 encompasses 148 amino acids and contains the entire N terminus, the A region (aa 186 to 464), and the first 49 amino acids of the post-A-region sequence (25). To evaluate whether known epitopes were conserved within the recombinant protein, we reacted $P1_{39–512}$ with several previously characterized P1-specific MAbs (1-6F, 4-9D, 4-10A, and 3-8D) (40–43). These MAbs were selected because they recognize epitopes that have been mapped to the N-terminal and central portions of the P1 protein (Fig. 1b). Three of these MAbs (1-6F, 4-9D, and 4-10A) were demonstrated previously to inhibit P1-mediated *S. mutans* adhesion to abiotic surfaces (42, 43). $P1_{39–512}$ was also subjected to a heat denaturation treatment to evaluate the presence of conformational epitopes recognized by the MAbs. The results presented in Fig. 2 show that all four anti-P1 MAbs recognized the untreated $P1_{39–512}$ antigen to some degree when tested by ELISA (Fig. 2a). Heat denaturation ablated recognition by MAbs 1-6F, 4-9D, and 4-10A but increased the reactivity of MAb 3-8D (Fig. 2b), which reacts within the A region and does not bind well to the intact native protein (9, 13, 14). Only MAb 3-8D reacted with denatured $P1_{39–512}$ in Western blots (Fig. 2c). Taken together, these results indicate that $P1_{39–512}$ preserves several different structural epitopes contained within the full-length native *S. mutans* protein. Unmasking of the 3-8D epitope by denaturation further confirms that untreated $P1_{39–512}$ achieves a native-like conformation, since this epitope is only partially accessible in the context of the folded molecule.

Recombinant $P1_{39–512}$ binds to immobilized SAG. We next assessed the functional properties of $P1_{39–512}$ by interactions with SAG. As can be seen in Fig. 2d, $P1_{39–512}$ binds to SAG immobilized on a BIAcore chip, as demonstrated by SPR. Heat denaturation of $P1_{39–512}$ eliminated its binding to immobilized SAG, suggesting that the SAG-binding activity is heat sensitive and dependent on structural aspects of the protein.

Characterization of antibody responses in mice immunized with $P1_{39–512}$ in combination with different adjuvants. In the next step, we evaluated whether the immunogenicity of $P1_{39–512}$ could be enhanced by s.c. administration to BALB/c mice in combination with three different vaccine adjuvants: alum, an LT derivative, and a *Salmonella* flagellin (FliC) (Fig. 3). Coadministration of adjuvants, particularly alum and LT, significantly increased the systemic IgG responses against full-length P1. After the third dose, titers were 2.6×10^4 ($P1_{39–512}$ plus alum), 2.4×10^4 ($P1_{39–512}$ plus LT), and 8.4×10^3 ($P1_{39–512}$ plus FliC), which were higher than that achieved in mice immunized with nonadjuvanted $P1_{39–512}$ (titer of 3.3×10^3) (Fig. 3a). Immunized mice maintained high anti-P1 serum IgG levels for at least 3 months after the last vaccine dose (data not shown). Analysis of the serum IgG subclass responses showed that immunization with $P1_{39–512}$ resulted in a balanced Th1/Th2 response (Fig. 3b). Mice immunized with $P1_{39–512}$ combined with adjuvants showed not only increased amounts of IgG1 but also larger amounts of IgG2a (alum and LT) and/or IgG2b (LT and FliC). Both IgG2a and IgG2b, but not IgG1, have been correlated with the antiadhesive activity of P1-specific antisera (49), suggesting that the efficacy of the antibodies would be affected by the incorporation of adjuvants. As expected, parenteral immunization of mice with the different vaccine formulations did not induce significant amounts of secretory IgA in saliva or feces (data not shown).

Effect of adjuvants on the avidity of anti-$P1_{39–512}$ antibodies and on the recognition of P1 on the surface of *S. mutans*. To determine whether the incorporation of adjuvants into the vaccine formulations altered the overall avidity of the induced antibodies, an ELISA-based ammonium thiocyanate dissociation assay was performed. There were no significant differences in the measured avidities of the anti-$P1_{39–512}$ antibodies raised in the different vaccination groups (Fig. 4a). We also measured the ability of the anti-$P1_{39–512}$ antibodies to bind to *S. mutans* whole cells, since this would be an important property of an effective vaccine. As illustrated in Fig. 4b, P1-specific antibodies from all four immunization groups bound to the surface of *S. mutans* cells from strain UA159. Antibodies raised in mice immunized with $P1_{39–512}$ admixed with alum displayed lower reactivity with *S. mutans* cells than did antibodies in samples collected from animals immunized with $P1_{39–512}$ only. In contrast, antibodies in sera collected from animals immunized with LT and FliC were more reactive with *S. mutans* than were those in the sera from mice immunized with $P1_{39–512}$. Similar results were observed with strain NG8 (data not shown), which is expected, since the P1 proteins from both strains share 97% identity, and in the relevant sequence (aa 39 to 512), this identity increases to 99%.

Adjuvant coadministration influences the specificity of P1-specific antibodies. In the next step, we tested whether adjuvant incorporation affects the epitope specificity of antibodies induced against $P1_{39–512}$. As shown in Fig. 4c, sera from mice vaccinated with the recombinant protein alone demonstrated similar reactivity with untreated and heat-denatured $P1_{39–512}$, suggesting that these antibodies were not directed against conformational determinants. Conversely, antibodies raised in animals immunized with $P1_{39–512}$ in combination with the tested adjuvants reacted

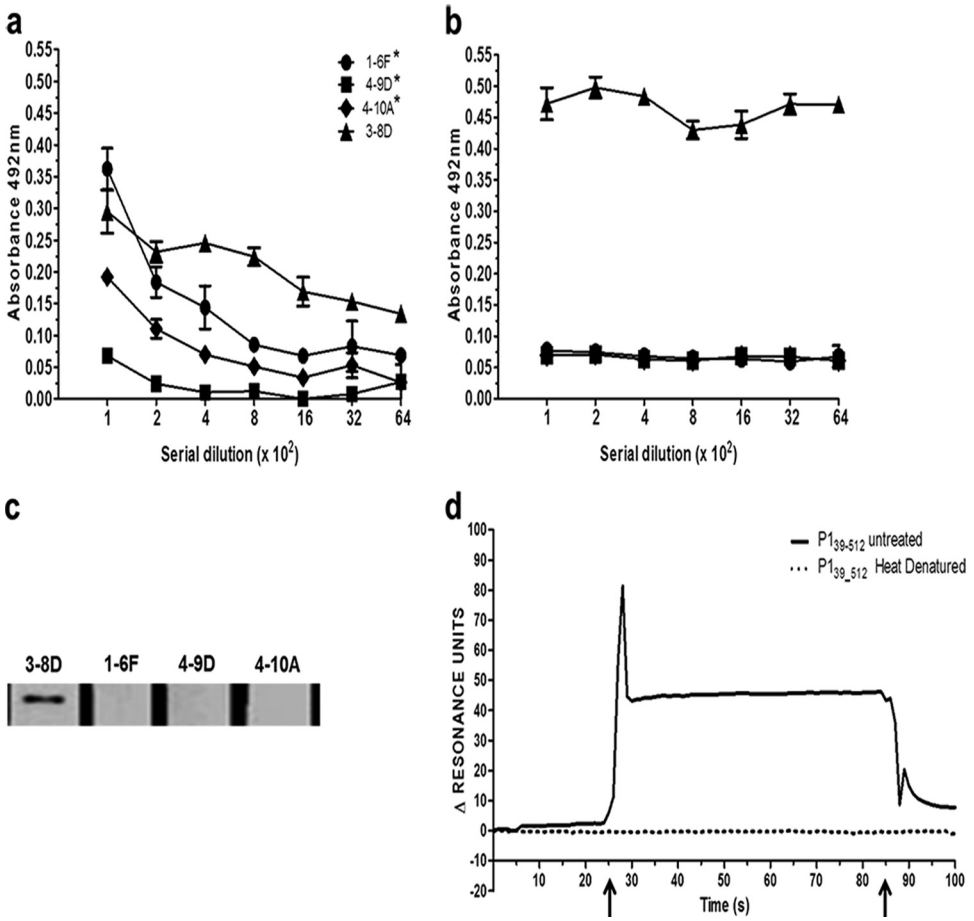

FIG 2 Recombinant $P1_{39-512}$ retains functional epitopes of the native *S. mutans* P1 protein. (a) Reactivity of $P1_{39-512}$ with MAbs that recognize epitopes localized to the N-terminal half of P1. Plates were coated with soluble $P1_{39-512}$ produced in recombinant *B. subtilis* strain LDV701 and reacted with 2-fold serial dilutions (beginning at 1:100) of MAb 1-6F, 4-9D, 4-10A, or 3-8D by ELISA. Asterisks indicate the anti-P1 MAbs that are strong inhibitors of *S. mutans* adherence. (b) Same as panel a but with reactions carried out with heat-denatured $P1_{39-512}$ as the immobilized antigen. (c) Reactivity of $P1_{39-512}$ with anti-P1 MAbs by Western blotting. Recombinant $P1_{39-512}$ was separated on a 7.5% SDS denaturing gel, blotted onto nitrocellulose, and then reacted with each MAb followed by goat horseradish peroxidase-conjugated anti-mouse IgG. (d) Binding of $P1_{39-512}$ to immobilized SAG monitored by surface plasmon resonance (BIAcore). The chip surface was coated with SAG and reacted with untreated or heat-denatured recombinant $P1_{39-512}$. Arrows indicate the beginning and end of the injection period.

more strongly with the nondenatured form of the protein. This observation suggests that coadministration of adjuvants contributed to the formation of additional antibodies against conformational determinants retained in $P1_{39-512}$.

In order to further investigate changes in epitope specificity of antibodies generated in mice immunized with $P1_{39-512}$ in combination with the adjuvants, we measured the reactivity of serum samples with several available P1-derived fragments that overlap the $P1_{39-512}$ sequence (Fig. 1a). The MA41 fragment corresponds to the A region (aa 185 to 472). It is recognized by MAb 3-8D (14), a weak inhibitor of immobilized SAG adherence (48). The LT1 fragment (aa 465 to 825) corresponds to the globular region of P1 that intervenes between the alanine- and proline-rich repeat segments. LT1 is recognized by MAb 1-6F, an antibody that is highly inhibitory of bacterial adherence to SAG (42, 49). The NR21 fragment (aa 465 to 825 fused to aa 987 to 1218) lacks the P region and was generated as a tool for previous epitope mapping studies (43). It is also recognized by the adherence-inhibiting MAb 1-6F. As expected, serum samples from all four groups were reactive with MA41. Coadministration of adjuvants significantly increased serum reactivity against MA41. In contrast, only serum samples from mice immunized with $P1_{39-512}$ admixed with alum, LTK4R, or FliC, but not those from mice immunized with $P1_{39-512}$ alone, recognized NR21. Only sera from mice immunized with $P1_{39-512}$ and LTK4R recognized LT1 (Fig. 4d). The gain of reactivity of antibodies against LT1, but not against NR21, in mice immunized with $P1_{39-512}$ plus LTK4R suggests that at least one epitope may be masked within the longer NR21 polypeptide. Given the highly complex structure of P1, it is not surprising that certain epitopes may or may not be formed or exposed depending on their context. Collectively, our results strongly suggest that incorporation of adjuvants within the vaccine formulation broadens the epitope specificity of the induced antibodies, including that against determinants recognized by MAbs known to inhibit the adhesive function of *S. mutans*.

Coadministration of adjuvants with $P1_{39-512}$ enhances the inhibition of *S. mutans* adhesion to immobilized SAG. Next, serum samples collected from immunized mice were tested for inhibition of *S. mutans* adherence to immobilized SAG as well as for inhibition of bacterial aggregation in the presence of soluble SAG (Fig. 5a). Sera from mice immunized with $P1_{39-512}$ admixed with alum, LT, or FliC all showed a significant improvement in their ability to inhibit bacterial adherence compared to those from mice immunized with $P1_{39-512}$ alone. Sera from mice immunized with

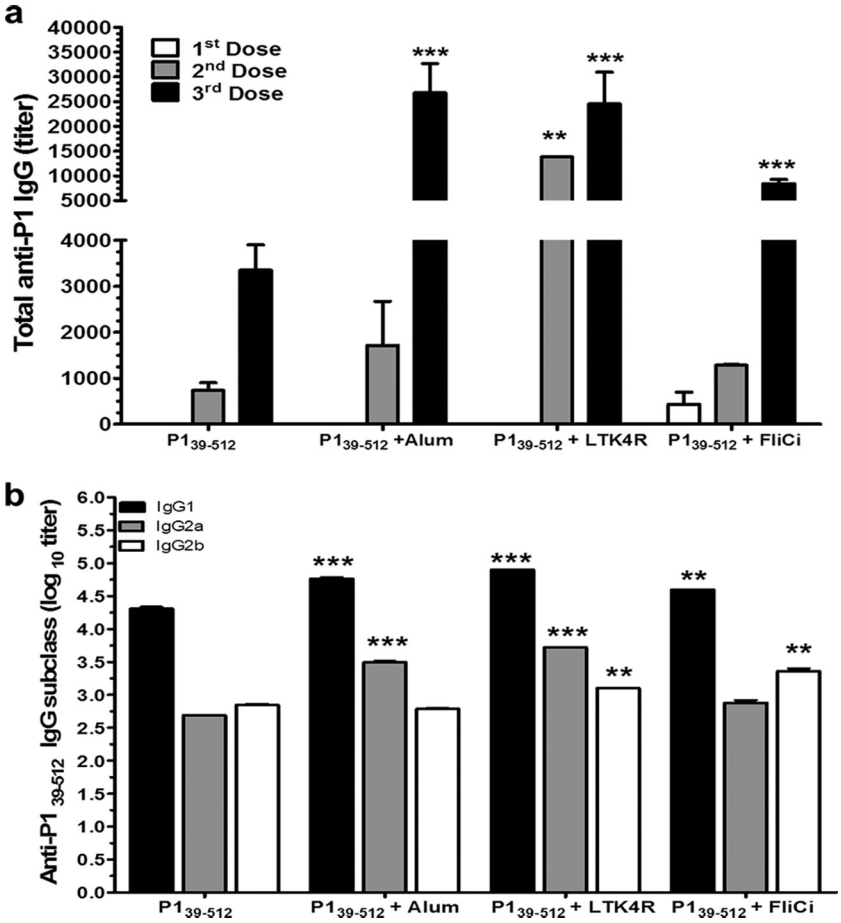

FIG 3 Induction of P1-specific serum IgG in mice immunized s.c. with $P1_{39-512}$ admixed with different adjuvants. (a) P1-specific serum IgG responses in BALB/c mice immunized with 3 vaccine doses containing 10 μg/dose of $P1_{39-512}$ alone or coadministered with aluminum hydroxide (alum) (12.4 μg/dose), LT (1 μg/dose), or *Salmonella* flagellin (FliC) (5 μg/dose). Serum samples were collected 2 weeks after each dose. ELISAs were performed on pooled samples by using purified full-length recombinant P1 as the immobilized antigen on the plate. (b) Anti-$P1_{39-512}$ IgG subclass responses measured in pooled sera of vaccinated mice following the third vaccine dose. Data are based on two independently performed immunization experiments and are expressed as means ± standard errors (**, $P < 0.01$; ***, $P < 0.001$).

$P1_{39-512}$ admixed with alum, but not those admixed with LTK4R or FliC, exhibited a moderate increase in inhibition of SAG-mediated bacterial aggregation, although this was not as pronounced as the increase in adherence inhibition observed for this group.

Adjuvants enhance the neutralizing capacity of anti-$P1_{39-512}$ antibodies *in vivo*. Considering that P1 is involved in the initial stages of colonization by *S. mutans*, we developed a murine oral colonization model to evaluate the protective potential of anti-P1 antibodies *in vivo*. First, we confirmed that early colonization of the tooth surface by *S. mutans* was dependent on the presence of P1. The wild-type strain, which produces P1, was detectable at significantly higher levels (~1.7×10^4 CFU) 6 h after inoculation than the *spaP*-negative mutant (PC3370) (~1.6×10^2 CFU), which lacks P1 (Fig. 5b). Also, mouse oral colonization by *S. mutans* was dependent on the presence of human saliva (data not shown). Next, to determine if antibodies raised against $P1_{39-512}$ alone, or admixed with adjuvants, could prevent colonization in this model, we preincubated *S. mutans* with sera collected from mice in the different immunization groups prior to introduction of the bacteria into the oral cavities of naive mice. *S. mutans* colonization was significantly reduced by pretreatment of bacteria with sera from all four groups, with the greatest reductions being seen with sera collected from mice immunized with $P1_{39-512}$ combined with alum or LTK4R. Sera from mice immunized with FliCi combined with $P1_{39-512}$ reduced *S. mutans* colonization to the same extent as did sera from mice immunized with $P1_{39-512}$ alone. These results indicate that antibodies raised in mice following immunization with $P1_{39-512}$, particularly in combination with alum or LTK4R, disrupt P1-dependent oral colonization of *S. mutans* in a murine model.

DISCUSSION

P1 is a widely studied antigen capable of triggering protective immunity against *S. mutans* infection and caries formation (21–23). Despite much promise, an effective vaccine against tooth decay is not yet available; therefore, identification of recombinant antigens capable of eliciting antibodies optimal for the blockage of bacterial adhesion to the tooth surface is critical. The *S. mutans* P1 protein is an exceptionally large and structurally complex protein for which a detailed framework of the key epitopes involved in bacterial adhesion is still incomplete. Generation of a recombinant P1 fragment preserving relevant immunological features of the native

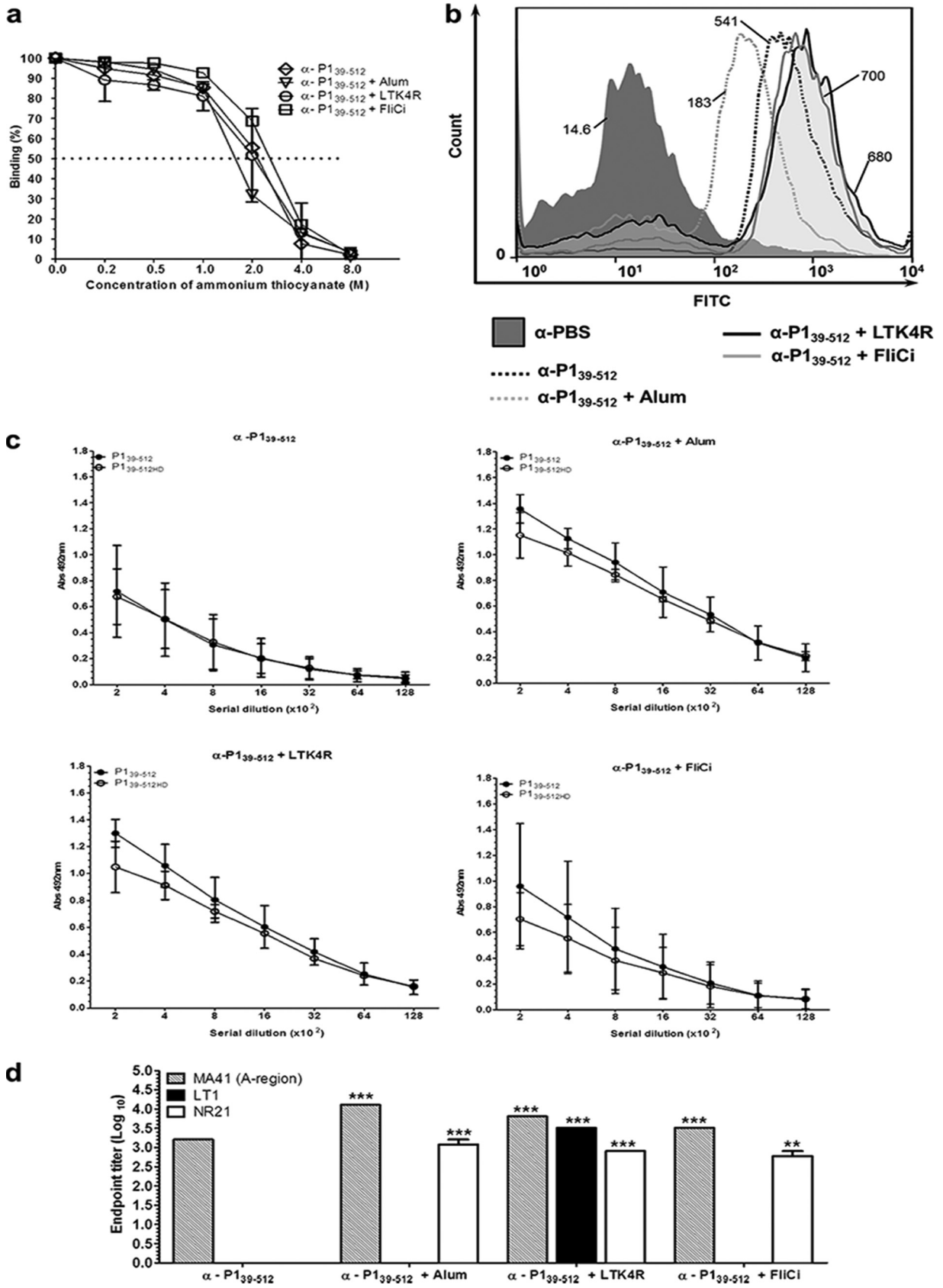

FIG 4 Determination of avidity, reactivity with the *S. mutans* cell surface, and epitope specificity of antibodies raised in mice immunized with $P1_{39-512}$ and the different vaccine adjuvants. (a) Antibody avidity was assessed by normalizing pooled serum samples from each group to achieve an absorbance of 1 on ELISA

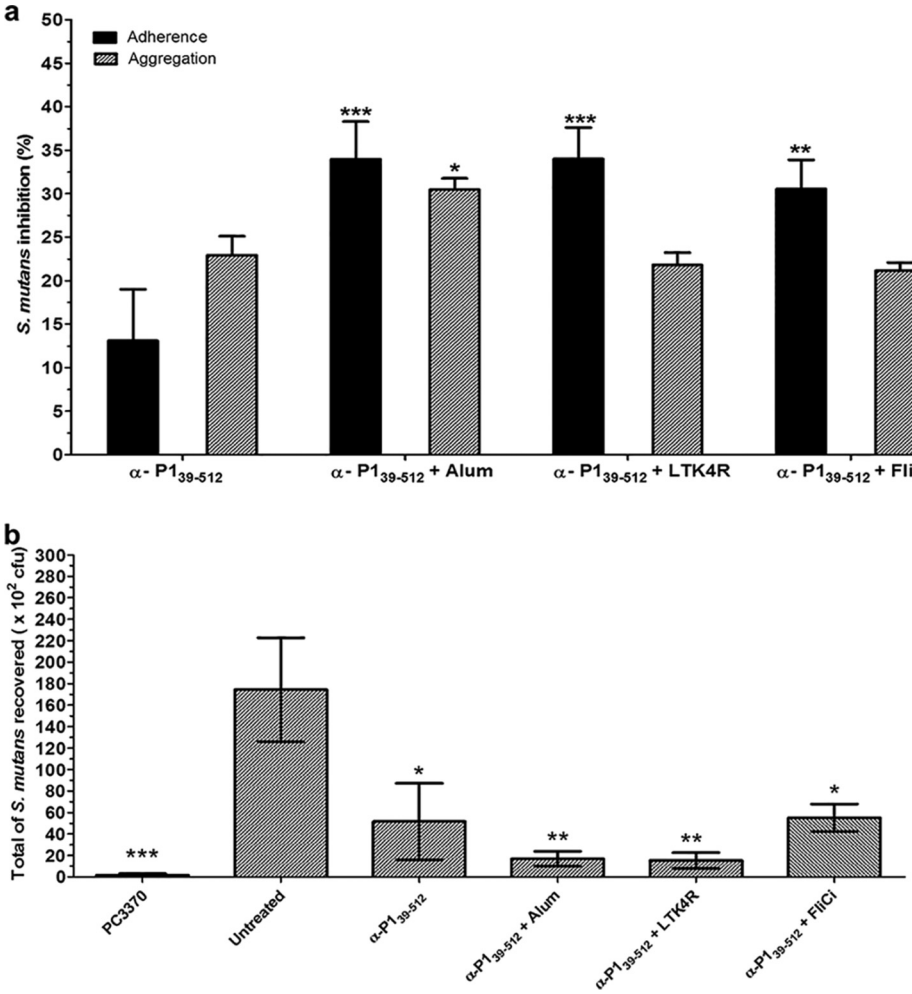

FIG 5 Blockage of the adhesive functions of *S. mutans* with anti-$P1_{39–512}$ antibodies. (a) Inhibition of SAG-mediated adhesion and aggregation of *S. mutans* cells by anti-$P1_{39–512}$ antibodies. Determination of adherence inhibition was carried out by surface plasmon resonance analysis. The percentage of adherence inhibition by immune serum samples from the indicated groups was determined by using the values obtained for *S. mutans* without serum as the reference. The percentage of inhibition of bacterial aggregation was calculated after incubation for 1 h in the presence of fluid-phase SAG and immune serum in comparison to *S. mutans* cells incubated without serum. Nonimmune mouse serum was used as a negative control, and the values were subtracted as the background. The results are based on data from at least in two independently performed experiments, and data represent means ± SEM. Statistically significant differences were determined in comparison to serum samples from mice immunized with $P1_{39–512}$ without adjuvant. (b) Inhibition of initial colonization of murine tooth surfaces with *S. mutans* by anti-$P1_{39–512}$ antibodies. BALB/c mice were pretreated with human saliva and then inoculated with *S. mutans* NG8 cells previously incubated with sera from mice immunized with $P1_{39–512}$ with and without adjuvants. Tooth swabs were collected 6 h after inoculation and plated onto Mitis-Salivarius medium plates containing bacitracin (0.2 U/ml) and sucrose (5%). *S. mutans* without preincubation with serum (untreated) and a P1-deficient mutant strain (PC3370) were used as positive and negative controls, respectively. Assays were performed with serum samples collected after the third dose. All sera were diluted 1/100. Data are expressed as the means ± SEM. Statistically significant differences were determined in comparison to the untreated group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

protein is an essential step for the development of an effective anticaries vaccine.

In the present study, we investigated functional, conformational, and immunological features of $P1_{39–512}$, a recombinant protein generated in *B. subtilis* cells that encompasses the saliva-binding region of the *S. mutans* P1 protein and adjacent sequences. Our results demonstrated that $P1_{39–512}$ displays conformational epitopes similar to those present in the native protein P1

plates coated with $P1_{39–512}$ and tested for dissociation with increasing amounts of ammonium thiocyanate, as described in Materials and Methods. The dotted line indicates 50% dissociation of antigen-antibody complexes. (b) Determination of reactivity of anti-$P1_{39–512}$ antibodies with native P1 protein on the surface of *S. mutans* cells by FACS analysis. Immunization groups are indicated. The median bacterial fluorescence intensity is indicated. (c) Determination of reactivity of serum samples with recombinant $P1_{39–512}$ protein by ELISA. $P1_{39–512}$ was used as a solid-phase antigen in two forms: untreated or heat denatured ($P1_{39–512HD}$). Immunization groups are indicated at the top. (d) Reactivity of anti-$P1_{39–512}$ antibodies with other truncated P1 fragments (Fig. 1a). Recombinant proteins representing the A region (MA41), the segment intervening between the A- and P-repeat regions (LT1), and a polypeptide representing LT1 fused to post-P-region sequence (NR21) were used as solid-phase-bound antigens in ELISAs. All experiments were performed with serum pools collected from mice following the third vaccine dose. Results are represented as endpoint titer values. Statistically significant differences were determined in comparison to serum samples from mice immunized with $P1_{39–512}$ without adjuvant (**, $P < 0.01$; ***, $P < 0.001$).

and also retains the ability to bind to human SAG. In addition, parenteral immunization of mice with $P1_{39-512}$ in combination with adjuvants not only affected the amounts of anti-P1 antibodies raised in vaccinated animals but also changed the IgG subclass differentiation and epitope specificity of the generated antibodies, particularly against conformational epitopes. More importantly, anti-$P1_{39-512}$ antibodies interfered with the adhesion of *S. mutans* to immobilized SAG, both *in vitro* and *in vivo*, and its protective potential was enhanced by the inclusion of adjuvants in the immunization schedule. Altogether, these results indicate that $P1_{39-512}$ preserves relevant features of the native *S. mutans* protein and should be regarded as a promising candidate for anticaries vaccine development.

The *S. mutans* P1 adhesin comprises a novel three-dimensional structure, and the appropriate folding of the protein is critical to its cell surface localization and function (16, 17, 48, 50). Our results indicate that even in the absence of the P region, the recombinant polypeptide $P1_{39-512}$ exhibits conformational features that mimic those of the native protein. This conclusion is based on the fact that the recombinant protein was recognized by three different anti-P1 MAbs, namely, 1-6F, 4-9D, and 4-10A, all of which recognize complex antigenic determinants of the P1 protein and inhibit bacterial adherence (41–43, 48, 49). Recognition by these antibodies, and the ability of $P1_{39-512}$ to interact with immobilized SAG, was lost after thermal denaturation of the protein, suggesting that epitope formation and functionality are dependent on structural features of the polypeptide. In contrast, the 3-8D epitope contained within the A region (9) is thought to be linear and is partially masked within the full-length molecule (14). Similarly, our results support the proposed nature of this epitope and demonstrate that it is also partially masked within the recombinant $P1_{39-512}$ protein, as evidenced by the increased reactivity of 3-8D after heat denaturation of this polypeptide.

Together, our data indicate that recombinant $P1_{39-512}$ produced in *B. subtilis* displays important conformational neutralizing epitopes and, therefore, sufficiently preserves the natural structure of the native protein. Furthermore, the N-terminal SAG-adhesive site of P1 appears to be located within an area spanning the third alanine-rich repeat and immediately adjacent to the post-A-region sequence (aa 386 to 512), since these residues are shared between $P1_{39-512}$ and the previously crystallized A3VP1 polypeptide (aa 386–874), which also binds immobilized SAG (15). In fact, Okuda and collaborators have also shown that a small peptide (aa 390 to 400) from *Streptococcus gordonii* is able to bind to salivary receptors and to prevent *S. gordonii* and *S. mutans* binding to saliva-coated hydroxyapatite (s-HA) in an *in vitro* assay (51). Together with our previous observations, the present data indicate that $P1_{39-512}$ preserves key epitopes recognized by antibodies capable of inhibiting P1-mediated adherence of *S. mutans* to SAG-coated surfaces.

The performance of subunit vaccines relies on the right combination of adjuvants, which enhance both the quantity and the quality of an induced immune response. Taking this into consideration, we evaluated the impact of three different adjuvants on the immunogenicity of $P1_{39-512}$ with regard to serum IgG titers, IgG subclass, antigen avidity, epitope specificity, and functional activity of induced antibodies. As expected, incorporation of adjuvants significantly augmented the total quantity of P1-specific IgG antibodies. In addition, incorporation of alum and LTK4R resulted in increases in antigen-specific IgG2a levels, while LKT4R and FliC promoted increases in IgG2b levels. Since the IgG2a and IgG2b subclasses have previously been correlated with an ability to neutralize the adhesive properties of *S. mutans* (41, 48), the increased amounts produced in mice immunized in conjunction with LT or flagellin would be expected to have a positive impact on the inhibition of bacterial adherence to SAG.

Another notable difference observed for the immune responses elicited in mice immunized with adjuvanted vaccine formulations was the altered epitope specificity of the induced antibodies. This was particularly evident in the recognition of heat-labile conformational epitopes associated with bacterial adherence and recognized by previously characterized MAbs capable of blocking the adhesion of *S. mutans*. In the absence of adjuvants, antibodies raised in mice immunized with recombinant $P1_{39-512}$ recognized predominantly heat-stable nondenaturable linear epitopes. Incorporation of adjuvants resulted in increased amounts of antibodies reacting with polypeptide MA41, corresponding to the A region. In addition, all of the adjuvants also increased reactivity against NR21, a fusion polypeptide that was generated in previous studies to assess the contribution of the P region to the adhesive function of P1 and that is recognized by MAb 1-6F but not the other MAbs in our panel (41). The increased reactivity against NR21 is consistent with the observed increases in IgG2a and IgG2b levels and the improved ability of the adjuvanted sera to inhibit *S. mutans* adherence to immobilized SAG. In previous studies, we found that 1-6F-like antibodies belong primarily to the IgG2a and IgG2b subclasses (36, 48). In addition, mice immunized with $P1_{39-512}$ in conjunction with LTK4R, but not the other combinations, also developed antibodies that recognize the LT1 polypeptide. The conformation-dependent adherence-inhibiting MAb 1-6F recognizes both LT1 and NR21, supporting the positive role of adjuvants in the generation of functional antibodies. Our results suggest that the adjuvant effect of heat-labile toxins favors the generation of antibodies against conformational determinants relevant for the function of the P1 adhesin. Previous studies employing the saliva-binding region (SBR) of P1 have also reported positive results when the immunogen was administered in combination with different LT derivatives, either as an isolated polypeptide or as a chimeric protein genetically fused to the B subunit of cholera toxin (52, 53).

Host defense against oral infection by *S. mutans* requires the induction of neutralizing antibodies that prevent or reduce bacterial adhesion to the enamel surface. As a mediator of sucrose-independent attachment, P1 is an important target of such antibodies (21, 22, 27, 52–55). To infer the potential protective effects conferred by immunization with $P1_{39-512}$, we determined if serum samples collected from vaccinated mice would reduce the initial adhesion of *S. mutans* to the dental surface of nonimmunized mice. Our results clearly demonstrated that anti-$P1_{39-512}$ antibodies raised in vaccinated mice interfere with this process. The inclusion of adjuvants, specifically alum and LTK4R, made the induction of antibodies capable of inhibiting initial oral colonization of mice by a cariogenic *S. mutans* strain even more effective. Thus, immunization with $P1_{39-512}$, especially when coadministered with adjuvants, represents a promising strategy for the generation of neutralizing antibodies that act both *in vitro* and *in vivo* to reduce *S. mutans* adherence, including adherence to saliva-coated teeth. Furthermore, our method to assess initial oral colonization of mice by *S. mutans* may be a useful and facile tool for those who

are interested in evaluating the efficacy of anti-*S. mutans* strategies.

In conclusion, our results indicate that $P1_{39-512}$ contains relevant target epitopes and induces adherence-inhibiting neutralizing antibodies that are effective *in vivo*. We provide evidence that adjuvants modulate the immune system to improve the quantity, and particularly the quality, of the host antibody response against *S. mutans* P1. Since serum IgG, which enters the oral cavity via the gingival crevice (21, 27–29), as well as salivary IgA (2, 4) contribute to immune protection against caries, the search for vaccine formulations allowing concomitant activation of a mucosal response is a priority. In addition, it may be possible to improve efficacy further by incorporation of relevant C-terminal target sequences that correspond to the second known adhesive domain of P1 (15, 16, 37), in conjunction with appropriate adjuvants and delivery vectors.

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