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Beneficial Immunomodulation by *Streptococcus mutans* Anti-P1 Monoclonal Antibodies Is Fc Independent and Correlates with Increased Exposure of a Relevant Target Epitope


We showed previously that deliberate immunization of BALB/c mice with immune complexes (IC) of the cariogenic bacterium *Streptococcus mutans* and mAbs against its surface adhesin P1 results in changes in the specificity and isotype of elicited anti-P1 Abs. Depending on the mAb, changes were beneficial, neutral, or detrimental, as measured by the ability of the serum from immunized mice to inhibit bacterial adherence to human salivary agglutinin by a BIACore surface plasmon resonance assay. The current study further defined changes in the host response that result from immunization with IC containing beneficial mAbs, and evaluated mechanisms by which beneficial immunomodulation could occur in this system. Immunomodulatory effects varied depending upon genetic background, with differing results in C57BL/6 and BALB/c mice. Desirable effects following IC immunization were observed in the absence of activating FcRs in BALB/c *Fcer1g* transgenic mice. mAb F(ab')2 mediated desirable changes similar to those observed using intact IgG. Sera from IC-immunized BALB/c mice that were better able to inhibit bacterial adherence demonstrated an increase in Abs able to compete with an adherence-inhibiting anti-P1 mAb, and binding of a beneficial immunomodulatory mAb to *S. mutans* increased exposure of that epitope. Consistent with a mechanism involving a mAb-mediated structural alteration of P1 on the cell surface, immunization with truncated P1 derivatives lacking segments that contribute to recognition by beneficial immunomodulatory mAbs resulted in an improvement in the ability of elicited serum Abs to inhibit bacterial adherence compared with immunization with the full-length protein. The *Journal of Immunology*, 2009, 183: 4628–4638.

*Streptococcus mutans* is the primary etiologic agent of dental caries in humans (1), a common infectious disease in the United States and worldwide. A number of virulence factors of *S. mutans* have been studied as vaccine candidates (2–5). The focus of the current study is a *M*~r~ 185,000 protein of *S. mutans* serotype c called P1. Originally identified as Ag I/II (6), and also called Ag B or PAc (7), it is a member of a family of structurally complex cell surface-anchored multifunctional adhesins. P1-like polypeptides are produced by almost all species of oral streptococci indigenous to the human oral cavity, and mediate interactions with salivary constituents, host cell matrix proteins, such as fibronecin, fibrinogen, collagen, and other oral bacteria (8). P1 contains a series of alanine-rich repeats (A region), a V region where most sequence variations between P1 from different bacterial strains are clustered, a series of proline-rich repeats (P region), and C-terminal sequences characteristic of wall and membrane-spanning domains of streptococcal surface proteins (Fig. 1A).

An important physiologic binding partner of P1 contained within the salivary pellicle is the large m.w. glycoprotein called salivary agglutinin (SAG) (9–12), now known to represent the human salivary scavenger protein gp340 (13). The interaction of P1 with salivary components is complex (14), and different regions of P1 are involved in its interaction with human SAG depending on whether SAG is in fluid phase or immobilized on a surface (9).

Humoral immunity against dental caries in animal models and naturally sensitized humans has been reported for many years (reviewed in Refs. 2 and 15). P1 (Ag I/II, PAc) has been studied in both active and passive immunization approaches, but a definitive correlate of protection particularly at the epitope level has not yet been fully elucidated. Numerous reports have stated that salivary and serum Abs, including those against P1, can be both protective or nonprotective, depending on the study (16–22). Collectively, these studies suggest that the fine specificity of the immune response is an important determinant in clinical outcome. There is long-standing and recent evidence that passively administered Ab may not be entirely passive, but can also have immunomodulatory effects (23–26). Deliberate immunization with Ag bound by Ab can result in suppression, enhancement, and differences in the elicited immune response, and exogenously applied Ab has been reported to act as a therapeutic agent by redirecting the host immune response (27). Numerous changes in immune responses against Ag coupled with mAbs have been documented (28–31), although the mechanism(s) mediating such changes is not completely understood and most likely overlaps and varies depending on the system.

Previous studies in our laboratory identified six different anti-P1 IgG1 mAbs that influence the anti-P1 response when they are bound to the surface of *S. mutans* whole cells and administered i.p.
to BALB/c mice as part of an immune complex (IC) (32–34). Differences in the immune response include changes in the ability of sera from IC-immunized mice to inhibit S. mutans adherence to SAG, measured using a whole-cell BIAcore surface plasmon resonance (SPR) assay, as well as changes in the specificity and isotype of anti-P1 Abs elicited in mice receiving IC compared with S. mutans alone. Anti-P1 mAbs are not equal in their ability to inhibit adherence of S. mutans to SAG or in their ability to modulate the immune response against P1. The minimal primary sequence of P1 currently known to achieve each cognate epitope has been characterized (Fig. 1B) (35–38).

Anti-P1 mAbs 6-11A, 3-10E, and 5-5D are beneficial modulators of humoral immunity in that they promote a polyclonal response more inhibitory of bacterial adherence when they are administered as part of an IC. mAb 4-10A also inhibits adherence and was initially found to be neutral with regard to its influence on the adherence-inhibiting response (34). Although in the current study we found that 4-10A demonstrates a notable prozone-like effect and promotes a beneficial response in a concentration-dependent manner.

It was the purpose of the current study to continue to identify specific changes in the murine serum response mediated by beneficial immunomodulatory anti-P1 mAbs and to evaluate factors that contribute to their desirable outcome. To that end, the roles of host genetic background, activating FcRs, and mAb Fc on immunomodulation were evaluated. The ability of serum from immunized mice to inhibit adherence of S. mutans to immobilized SAG and to compete for binding of the known adherence-inhibiting mAb 1-6F, and the isotype composition of 1-6F-like Abs were used as measures of specific immunomodulatory changes resulting from IC immunization. In addition, the ability of an immunomodulatory mAb to influence exposure of the 1-6F epitope on the bacterial surface was explored. Lastly, to confirm whether desirable effects may result from a destabilizing effect on protein structure and an enhanced response against relevant target epitopes that may be revealed upon binding of beneficial anti-P1 mAbs to S. mutans, mice were immunized with truncated P1 variants that had been engineered to lack segments of P1 that contribute to their complex discontinuous epitopes.

**Materials and Methods**

**Bacterial strains, plasminids, and growth conditions**

Serotype c S. mutans strain NG8 was grown aerobically to stationary phase for 16 h in Todd-Hewitt broth supplemented with 0.3% yeast extract (BBL). The Escherichia coli host strains used to express recombinant P1 polypeptides were DH5α (Invitrogen) and M15 (pREP4) (Qiagen). E. coli strains were grown aerobically at 37°C with vigorous shaking in Luria-Bertani broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) supplemented with ampicillin (50–100 μg/ml) or kanamycin (25–50 μg/ml), as appropriate. Plasmids pCR2.1 (Invitrogen) and pMalp (New England Biolabs) were used as cloning and expression vectors.

**Construction and purification of P1 polypeptide NR21**

Plasmid pNR21 was derived by PCR amplification, as described (36), using forward primer 5′-GGGAGATTCTGAATTAGGCACGAGCATTC-3′ and reverse primer 5′-GGGGTTCAAGATATGTTTGCATGCTCT-3′, with the spap-derived P region deletion construct pCG2 (39) plasmid DNA as the template. spap is the gene that encodes P1. Underlined sequences indicate SacI and SalI restriction sites engineered into the forward and reverse primers, respectively, and were included for subsequent cloning into pMal-p and expression of the P1-derived polypeptide as a fusion partner with maltose-binding protein (MBP). NR21 was initially constructed to evaluate the contribution of the P region and sequences flanking it to epitopes recognized by anti-P1 mAbs. E. coli containing pNR21 was grown, as described (36). Overnight cultures were diluted 1/100 into fresh medium containing ampicillin (50–100 μg/ml), grown with shaking to OD600 of 0.45–0.6, and induced for 3–5 h at 37°C with 0.1–0.5 mM isopropyl-β-D-thiogalactoside (Fisher). The fusion protein was affinity purified from E. coli lysates by column chromatography using amylase resin (New England Biolabs), according to the manufacturer’s protocol.
Mice

Six- to 8-wk-old female BALB/c and C57BL/6 mice were purchased from Charles River Laboratories. Four 12-wk-old male and female Fcerlg (FcRy) transgenic mice were purchased from Taconic Farms. Mice were housed in biosafety level 2 facilities under infectious disease conditions and fed a standard diet.

Source of Abs

Anti-P1 mAbs were obtained from previously established hybridomas made in our laboratory (40). All mAbs used in this study were purified by column chromatography from ascites fluid using an ImmunoPure (A Plus) IgG Purification Kit (Pierce), according to the manufacturer’s protocol. Peroxidase-labeled and unlabeled secondary mAb reagents were obtained from Southern Biotechnology Associates.

Pepsin digestion of mAbs and purification of F(ab’)2

mAbs 5-5D and 6-11A were buffer exchanged into 0.2 M sodium acetate adjusted to pH 4.0 using glacial acetic acid, diluted or concentrated to ~2.0 mg/ml, and 0.1 mg/ml pepsin (Sigma-Aldrich) dissolved in the same buffer was added to achieve an enzyme:Ab ratio of 1:20. Samples were incubated at 37°C for 24–72 h, followed by addition of 2 M Tris base until the pH reached 7.0–8.0, and then dialyzed overnight into 1X PBS, pH 8.0. The digestion mixtures were delected of Fc fragments and residual undigested mAb by size exclusion on an Imobead (A Plus) IgG Purification Kit (Pierce), according to the manufacturer’s instructions. The protein A column was equilibrated with binding buffer, and the digested sample was dialyzed 1/1 in binding buffer and applied. Flow through and the first wash containing Fc-free F(ab’)2 were collected. Final purification of F(ab’)2 was performed on a Bio-s elect 250 gel filtration column (Bio-Rad). Purity of F(ab’)2 was verified by Western blot using anti-Fc-, anti-γ-chain-, anti-α-chain-, and anti-δ-chain-specific secondary Abs (Southern Biotechnology Associates).

Immunizations and sample collections

Groups of six mice were immunized i.p. with ~1.5 × 10⁹ CFUs of S. mutans in 150 μl of PBS or the same amount of S. mutans coated with either a saturating (high) or 0.1X subsaturating (low) concentration of each mAb (34) or with mAb-derived F(ab’)2 (3). The dilution of mAb or F(ab’)2 necessary to saturate an equal volume of the standardized concentration of bacterial cells was predetermined by serial titration and dot blot analysis. In the case of 4-10A, a dilution of 1/2000 of 3 mg/ml purified mAb represented a 0.1X subsaturating Ab concentration, and S. mutans cells were reacted with 2-fold serial dilutions of mAbs beginning at that point. To exclude the possibility of anti-Id effects and to control for other potential effects of mAbs that might occur independently of Ag, additional control groups of mice received mAb alone at the concentration required to saturate the density of bacteria. Sera from these mice were not reactive with P1 or S. mutans, nor were pre-immune sera from experimental groups or sera from negative control mice that received buffer only (data not shown). Mice were bled 1 wk before the first inoculation, immunized on days 0 and 14, and exsanguinated on days 30–40. Interim bleeds were also taken on day 7 in some cases. All experiments were performed with terminal serum collections, unless stated otherwise.

For experiments with P1 and truncated derivatives, groups of five mice were immunized i.p. with emulsified polycarboxamide or slices containing recombinant full-length P1 (CG14), or with recombinant P1 polypeptides CK1, CK2 (38), or NR21 (this study) expressed as fusion proteins with MBP. Negative control groups received MBP alone, polycarboxamide only, or PBS alone. E. coli containing pCG14, pCK1 and pCK2, pNR21, or pMAL-p vector alone was grown, as described (36). Overnight cultures were diluted 1/100 in fresh medium containing ampicillin (50–100 μg/ml) for CK1, CK2, NR21, and MBP control, or containing ampicillin (50–100 μg/ml) and kanamycin (25 μg/ml) for CG14, grown with shaking to OD₆₀₀ of 0.45–0.6, and induced for 3–5 h at 37°C with 0.1–0.5 mM isopropyl β-D-thiogalactoside (Fisher Scientific). Cells were harvested by centrifugation and boiled in SDS sample buffer, and lysates were loaded onto 7.5% SDS-polyacrylamide gels. Gels were negatively stained with 0.3 M cupric chloride for 5 min and then rinsed with water. Negative-stained bands of the correct size and corresponding to ~10 μg of total protein were excised and destained with 0.25 M EDTA and 0.25 M Tris (pH 9) by incubating three times for 10 min at room temperature with a final exchange into PBS. The gel slices containing each recombinant protein were emulsified into ~1 ml of PBS, and 100 μl was used to immunize individual mice. Mice were bled 1 wk before the first inoculation, immunized on days 0 and 14, and exsanguinated on day 36. All immunization experiments were performed under the guidelines and approved by the University of Florida Institutional Animal Care and Use Committee.

BIACore assay of S. mutans adherence to SAG

Adherence of S. mutans whole cells to human SAG immobilized on a CM3 sensor chip (GE Healthcare) was measured using the BIACore 3000 machine (BIACore), as previously described (33). SAG was prepared by a modification of the technique of Rundegren and Arnold (9, 41). Adherence of S. mutans cells pretreated with serum from mice immunized with bacteria alone vs those immunized with IC was compared in each experiment.

Western blot reactivity of anti-P1 Abs with NR21 and full-length P1

Recombinant full-length P1 (39) or recombinant polypeptide NR21 was electrophoresed on replicate 7% SDS-polyacrylamide preparatory slab gels and electroblotted onto nitrocellulose filters. Filters were blocked with PBS containing 0.3% Tween 20 and cut into strips, and replicate strips were reacted with each of the 11 different anti-P1 mAbs. Blot strips were washed, reacted with affinity-purified peroxidase-labeled goat anti-mouse IgG-specific Ab (Southern Biotechnology Associates), and developed with 4-chloro-1-naphthol substrate solution. Control blot strips were reacted with secondary Ab only (data not shown).

Biotin labeling of mAb 1-6F and competition ELISA

Approximately 1 mg of purified anti-P1 mAb 1-6F was labeled with biotin using EZ-Link Biotin-LC-Hydrazide (Pierce), according to the manufacturer’s protocol. ELISA plate wells were coated with S. mutans whole cells (~10⁵ CFU/well) (40) in carbonate-bicarbonate buffer (pH 9.6). To determine the optimal dilution for each competition experiment, immune sera from S. mutans or IC-immunized mice were serially diluted 3-fold beginning at 1/100 and added to the wells, followed by biotin-labeled mAb 1-6F, and incubated at 37°C for 2 h. Plates were washed, and avidin-HRP conjugate (Pierce) was applied to the wells for 30 min at room temperature. Plates were washed again and developed with 0.1 M o-phenylenediamine dihydrochloride containing 0.012% hydrogen peroxide in 0.01 M phosphate citrate buffer. Optimal working dilutions ranged from 1/500 to 1/900. Percentage of inhibition of biotin-labeled mAb 1-6F binding was calculated as percentage of inhibition = (OD₄₅₀ direct binding of biotin-labeled 1-6F – OD₄₅₀ experimental well/OD₄₅₀ direct binding of biotin-labeled 1-6F) × 100. Control wells contained nonlabeled mAb 1-6F and avidin-HRP.

Quantitative subclass ELISA

Serum samples were assayed for anti-S. mutans or anti-P1 polypeptide NR21 IgG1, IgG2a, and IgG2b isotype Abs by quantitative subclass ELISA. Sera from C57BL/6 mice were assayed for IgG2c instead of IgG2a (Southern Biotechnology Associates) plates wells were coated with NGS whole cells or 200 μg/ml purified recombinant NR21 in carbonate-bicarbonate buffer (pH 9.6). Mouse sera were serially diluted 2-fold beginning at 1/50 and added to the wells. Ab reactivity was detected using affinity-purified peroxidase-labeled goat anti-mouse IgG1, IgG2a, IgG2b, or IgG2c subclass-specific Abs (Southern Biotechnology Associates) at a 1/1000 dilution. Plates were developed with 0.1 M o-phenylenediamine dihydrochloride containing 0.012% hydrogen peroxide in 0.01 M phosphate citrate buffer. The concentration of anti-S. mutans or anti-NR21 IgG subclass Abs were calculated by interpolation on standard curves generated using purified mouse subclass reagents (Southern Biotechnology Associates).

Results

The genetic background of the host affects immunomodulation by anti-P1 mAbs

The protective efficacy of exogenously administered Abs has been shown to differ depending on the genetic background of the murine host and underscores the potential complexity of Ab-mediated effects on the subsequent adaptive immune response (43). To determine whether immunomodulation by anti-P1 mAbs is influenced by genetic background, immunization experiments were performed in BALB/c and C57BL/6 mice. Mice were immunized in parallel with uncoated S. mutans whole cells or with IC containing mAbs 6-11A or 5-5D at saturating or 0.1X subsaturating concentrations. As observed in our previous studies, BIACore SPR assays demonstrated that sera from BALB/c mice immunized with ICs containing mAbs 6-11A and 5-5D were increased in their ability to interfere with bacterial adherence to immobilized SAG; however,
the results were not identical in C57BL/6 mice treated in an identical manner (Fig. 2A).

The sera from the immunized BALB/c and C57BL/6 mice were also examined for changes in the isotype composition of the anti- S. mutans response. To evaluate the immune response in these mice early after exposure to Ag, sera collected 7 days after primary immunization were assayed by quantitative ELISA. Compared with serum from mice that received S. mutans alone, the serum from BALB/c mice immunized with ICs containing mAb 6-11A showed an increase in anti-S. mutans IgG1 and IgG2b at both mAb concentrations, and in IgG2a at the high mAb concentration (Fig. 2B). Again, the results in the C57BL/6 mice were different from those in the BALB/c mice, with the most pronounced effect seen with IgG1 at the low mAb concentration in the C57BL/6 mice. The sera from BALB/c mice immunized with ICs containing mAb 5-5D also contained increased levels of anti-S. mutans Abs of the IgG2a and IgG2b isotypes compared with the serum from the S. mutans-only immunized mice, whereas the sera from the C57BL/6-immunized did not demonstrate the same 5-5D mAb-mediated effects (Fig. 2C).

Several anti-P1 mAbs inhibit the binding of S. mutans to immobilized SAG (23, 34). Therefore, one would predict that the sera from 6-11A and 5-5D IC-immunized mice that had demonstrated an increased ability to inhibit bacterial adherence would also contain increased levels of Abs that recognize similar epitopes as adherence-inhibiting mAbs. To explore this possibility, the ability of the sera from S. mutans and IC-immunized BALB/c and C57BL/6 mice to compete with the adherence-inhibiting mAb 1-6F for binding to P1 on the surface of S. mutans whole cells was compared by competition ELISA. Sera from 6-11A and 5-5D IC-immunized

FIGURE 2. Host genetic background affects beneficial immunomodulation. A, BIAcore SPR analysis of S. mutans adherence to immobilized SAG. Sensograms show the binding of S. mutans to immobilized SAG in the presence of serum from BALB/c or C57BL/6 mice immunized with S. mutans alone or with IC containing mAbs 6-11A or 5-5D at high (saturating) or low (0.1× subsaturating) concentrations. B, Evaluation of IgG subclasses reactive with anti-S. mutans whole cells. Sera collected 7 days after primary immunization from BALB/c or C57BL/6 mice immunized with S. mutans alone (diamonds), IC containing mAb 6-11A at the higher concentration (squares), or lower concentration (triangles) were evaluated by quantitative ELISA. C, Same as B, except that mAb 5-5D was used in the experiment. D, A competition ELISA was used to determine the level of mAb 1-6F-like Abs in the serum of BALB/c and C57BL/6 mice immunized with ICs of mAb 6-11A and 5-5D compared with S. mutans alone. The percentage of inhibition of binding of biotin-labeled 1-6F to S. mutans by sera from mice immunized with bacteria alone ( ), high mAb concentration IC ( ), and low mAb concentration IC ( ) is indicated. Results are expressed as mean ± SEM, and statistical significance compared with serum from S. mutans-only immunized mice is indicated by p values. Data are representative of at least three independent experiments. Statistical analysis was performed using GraphPad Prism 4.0, and analysis included one-way ANOVA.
BALB/c mice, at the high and low mAb concentrations, respectively, but not the C57BL/6 mice, demonstrated increased levels of Abs capable of competing with biotin-labeled 1-6F for S. mutans binding over the serum from mice that had received bacteria alone (Fig. 2D).

These results confirmed our previous findings that 6-11A and 5-5D redirect the humoral immune response in BALB/c mice toward one of increased efficacy in terms of the ability of elicited Abs to functionally inhibit bacterial adherence to a known receptor. We also extended these findings to identify mAb-mediated changes in the IgG subclass composition of anti-S. mutans serum Abs during the initial immune response, and we further characterized the change in Ab specificity that accompanied the change in biological activity. That the same effects were not observed in the C57BL/6 strain indicates that the mAbs alter the subsequent immune response by a mechanism that is influenced by the genetic background of the host.

Further evaluation of competition against mAb 1-6F by sera from IC-immunized mice

With the new information that immunization with IC containing mAbs 6-11A and 5-5D promotes a polyclonal immune response that contains increased levels of Abs capable of competing for S. mutans binding with biotin-labeled 1-6F, additional competition experiments were undertaken. Previously, mAbs 6-11A, 3-10E, 5-5D, 1-6F, 4-9D, and 4-10A had all been tested for immunomodulatory activity when administered to BALB/c mice as part of an IC. Sera from the 6-11A, 3-10E, and 5-5D, but not the 1-6F, 4-9D, or 4-10A IC-immunized mice had demonstrated an increased ability to inhibit S. mutans binding to immobilized SAG by BLAcore assay (32, 34). Therefore, sera stored from these prior experiments were tested for their ability to compete for S. mutans binding with biotin-labeled mAb 1-6F.

The sera from the mice that had been immunized with IC containing mAbs 6-11A, 3-10E, and 5-5D all demonstrated significantly higher levels of Abs able to compete with mAb 1-6F for binding to S. mutans compared with sera from S. mutans-immunized mice (Fig. 3). In contrast, sera from mice that had been immunized with IC containing those mAbs shown not to promote an adherence-inhibiting response did not exhibit an increase in 1-6F-like Abs. These data demonstrate a link between the previously identified beneficial immunomodulatory mAbs and the promotion of an enhanced response against a relevant target epitope.

Activating FcRs are not required for beneficial immunomodulation by an anti-P1 mAb

Major mechanisms by which Ab has been reported to exert immunomodulatory effects are by way of promotion of uptake of Ag via FcRs on APCs and/or differential engagement of stimulatory vs inhibitory FcRs (29). To evaluate whether FcRs play a critical mechanistic role in anti-P1 mAb-mediated effects, an immunization experiment was conducted in mice lacking activating FcRs. The three receptors known to bind IgG and activate an immune response, FcγRI, FcγRIII, and FcγRIV, all depend upon a common γ-chain for signaling through an ITAM. The FcγR-targeted mutation in the Fcer1g transgenic mouse lacks a functional γ-chain (44). BALB/c background Fcer1g mice were immunized with S. mutans alone or with IC containing mAb 5-5D at either saturating or 0.1× subsaturating concentrations. Sera from both groups of IC-immunized mice were better able to interfere with adherence of S. mutans to immobilized SAG compared with mice that were immunized with S. mutans alone and demonstrated that activating FcRs are not necessary for the ability of the mAb to promote this desirable response (Fig. 4A). Furthermore, the sera from the IC-immunized mice demonstrated increased competition of biotin-labeled mAb 1-6F binding to P1 on the surface of S. mutans compared with the serum from S. mutans-immunized mice (Fig. 4B).

Because prior studies in our laboratory had demonstrated a statistically significant correlation between the ability of serum from IC-immunized mice to inhibit bacterial adherence to immobilized SAG and the levels of IgG2a and IgG2b reactive with S. mutans (34), we also wished to evaluate the isotype of Abs that were able to compete with biotin-labeled 1-6F for binding and to determine whether IgG2a and/or IgG2b subclasses were among those increased. To that end, a quantitative subclass ELISA was used, utilizing as the Ag recombinant P1 polypeptide NR21 that is recognized by mAb 1-6F, but none of the other anti-P1 mAbs in our panel (Fig. 1, C and D). Increased levels of anti-NR21 IgG1, IgG2a, and IgG2b were observed in the sera of mAb 5-5D IC-immunized Fcer1g mice compared with the serum from S. mutans-immunized Fcer1g mice and were most notable for the IgG2b isotype. Taken together, these results indicate that desirable immunomodulatory effects of a beneficial anti-P1 mAb can occur independently of the presence of activating FcRs.

The Fc portion of anti-P1 mAbs is not required for immunomodulation

Despite ruling out an apparent role of activating FcRs in the mechanism underlying beneficial immunomodulation by anti-P1 mAbs,
we wished to rule out the necessity of mAb Fc altogether to determine whether complement activation and subsequent uptake of the IC via complement receptors or engagement of inhibitory FcRs might be involved in our system. Therefore, immunization experiments were performed using IC containing F(ab')2 of mAbs 6-11A and 5-5D in place of intact mAbs. Sera from mice immunized with S. mutans alone or with IC containing high (saturating) or low (0.1 \times \text{sub saturating}) mAb concentration (triangles), or IC containing the low (0.1 \times \text{sub saturating}) mAb concentration (circles) is indicated. Results are expressed as mean \pm SEM, and statistical significance compared with serum from S. mutans-only immunized mice is indicated by p values. C. Quantitative anti-NR21 IgG subclass ELISA. Levels of anti-NR21-specific IgG1, IgG2a, and IgG2b subclass Abs were measured in sera collected from FcεR1g transgenic mice immunized with S. mutans alone (diamonds), or with IC containing mAb 5-5D at the high saturating (squares) or low 0.1 \times \text{sub saturating concentration} (triangles). Results are expressed as mean \pm SEM, and statistical significance compared with serum from S. mutans-only immunized mice is indicated by p values. Data are representative of at least three independent experiments. Statistical analysis was performed using GraphPad Prism 4.0, and analysis included one-way ANOVA.

Re-evaluation of the immunomodulatory properties of mAb 4-10A and exposure of the 1-6F epitope

A common denominator of the three known beneficial immunomodulatory mAbs 6-11A, 3-10E, and 5-5D is shared structural features of P1 that contribute to their complex conformationally dependent epitopes. All three of their cognate epitopes require an interaction of the discontinuous A and P regions of P1, with pre-A region sequence contributing to the epitopes of 6-11A, 5-5D, and 3-10E, and post-P region sequence also contributing to the epitope recognized by 3-10E (35-38). mAb 4-10A is similar to these mAbs in its requirement for the A region/P region interaction; however, it had previously been found to be neutral in its ability to promote the desirable bacterial adherence-inhibiting response (34, 45). Because prozone-like effects of exogenously administered Abs have been observed in ours and other systems and desirable results may require lower rather than higher amounts of Ab (23, 34, 46), 4-10A was re-evaluated in murine immunization experiments over a broader concentration range within the IC.

BALB/c mice were immunized with S. mutans only or S. mutans reacted with serial 2-fold dilutions of mAb 4-10A beginning at a 0.1 \times \text{sub saturating coating concentration}, and a pronounced prozone-like effect was observed (Fig. 6A). When tested by BIAcore SPR assay, the sera from mice immunized with S. mutans IC containing the intermediate concentrations of mAb were clearly able to interfere with bacterial adherence to SAG better than sera from mice that received S. mutans alone or mice that received IC containing the higher or lower concentrations of mAb (Fig. 6A). Optimal effects of sera from mice immunized with IC containing intermediate concentrations of mAb were also observed in other experiments. These included an increased ability of sera collected as early as 7 days postprimary immunization to compete for
S. mutans binding with biotin-labeled mAb 1-6F (Fig. 6B) and increased levels of anti-NR21 Abs of all three IgG isotypes, with IgG1 and IgG2a being the most pronounced (Fig. 6C).

Although mAb 1-6F is the only anti-P1 mAb in our panel to react with the P1-derived NR21 polypeptide, this mAb is not strongly reactive with purified full-length P1 (38), suggesting that its epitope is partially masked within the context of the entire molecule (Fig. 1D). Because all the sera from mice immunized with IC that contained beneficial mAbs, including 4-10A at intermediate concentrations, also contained increased levels of 1-6F-like Abs, we hypothesized that a mAb-mediated alteration in P1’s immunogenicity in vivo would be reflected by a detectable change in exposure of the 1-6F epitope on S. mutans surface-localized P1 in vitro. To test that possibility, a modification of the 1-6F competition...
ELISA was used; however, rather than inhibition of binding of the biont-labeled mAb to S. mutans whole cells, the measured end point was enhancement of 1-6F reactivity. When serial 2-fold dilutions of mAb 4-10A were reacted with S. mutans before addition of biont-labeled 1-6F, a greater than 100% increase in 1-6F (Fig. 6D) binding was observed in the same 4-10A concentration range as that shown to promote the formation of 1-6F-like Abs in the immunization experiments (compare Fig. 6B).

Immunization with truncated P1 elicits Abs better able to inhibit S. mutans adherence

To confirm that structural alteration of P1 can result in an improvement in the functional activity of elicited Abs, mice were immunized with full-length P1 compared with several truncated variants. These included polypeptides NR21, CK1, and CK2 (see Fig. 1). NR21 achieves the 1-6F epitope, but lacks the A and P regions, and, as stated above, is not recognized by any other anti-P1 mAbs. CK1 and CK2 lack the post-P region and varying degrees of pre-A region sequence, respectively. Pre-A region sequence encompassing amino acid residues 84–185 was shown previously to contribute to the epitopes recognized by the beneficial mAbs 6-11A and 5-5D (38). This segment is essential for formation of the 3-10E epitope (38); an interaction between the pre-A and post-P region (residues 964–1218) is required for achievement of native-like structure and recognition by mAb 3-10E (35). The binding of mAb 1-6F to CK1 and CK2 is greatly enhanced compared with the full-length recombinant P1 polypeptide CG14 (38), suggesting that the pre-A and post-P region interaction contributes to the conformation of P1, in which the 1-6F epitope is partially masked.

Compared with CG14, sera from mice that were immunized with CK1 or CK2 showed a substantial increase in their ability to inhibit adherence of S. mutans to immobilized SAG when evaluated by BIAcore assay (Fig. 7A). The degree of bacterial adherence in the presence of serum from sham-immunized PBS buffer-only mice is also shown. Despite the notable difference in functional activity, sera from the CK1 and CK2 groups of mice were similarly reactive to that from the CG14 group when tested against S. mutans whole cells by ELISA (Fig. 7B). This implies that the functional difference in adherence inhibition stemmed from a change in Ab specificity. The ability of sera from the different groups to inhibit binding of biont-labeled 1-6F to S. mutans whole cells by competition ELISA is shown in Fig. 7C. Serum from mice immunized with NR21 was not inhibitory of bacterial adherence (Fig. 7A), did not react with S. mutans whole cells (Fig. 7B), and was not an efficient competitor of 1-6F binding to the bacterial cell surface (Fig. 7C). This indicates that the NR21 polypeptide is not an effective immunogen, although it can serve as an appropriate Ag for detection of 1-6F-like Abs in immunoassays. In addition to 1-6F, the CK1 and CK2 polypeptides are both recognized by two additional adherence-inhibiting anti-P1 mAbs, 4-9D and 4-10A. The 4-10A epitope is completely reconstituted by interaction of separate fragments corresponding to the A and P regions, whereas the 4-9D epitope cannot be reconstituted in trans and is destroyed when P1 is disrupted between amino acid residues 464 and 465 (38). Hence, optimal immunogenicity appears to represent a balance between preservation of sufficient conformational complexity to reconstitute relevant target epitopes, while disrupting the native structure such that immunodominance is shifted toward a more effective response.
The overall goal of this study was to present a method of improving the efficacy of an immune response by shifting the response toward more effective, but potentially subdominant target epitopes. The immune response involves the nature of the Ag-Ab interaction necessary for formation of their cognate epitopes, implying that the mechanism responsible for their redirection of the response could be changed for the better. As a marker of biological function and activity against a known virulence attribute, we measured the ability of elicited Abs to interfere with P1-mediated adherence of \textit{S. mutans} to the SAG receptor normally present within the human salivary pellicle.

The anti-P1 mAb 1-6F inhibits adherence of \textit{S. mutans} to immobilized SAG, and increased levels of 1-6F-like Abs were consistently detected in the sera of animals immunized with IC containing beneficial immunomodulatory mAbs. In keeping with our previous results that demonstrated that adherence inhibition correlated with anti-\textit{S. mutans} Abs of the IgG2a and IgG2b isotypes (34), anti-1-6F-like Abs of either or both of these subclasses were also increased in the sera from IC-immunized mice that demonstrated increased functional activity in the BIAcore SPR assay.

Broadly classified, the ways that Ab is known to modulate the host immune response when it is bound to its specific Ag as part of an IC include Fc-dependent mechanisms such as increased uptake by APCs via FcRs, engagement of stimulatory or inhibitory FcRs on APCs (29, 31, 47–50), as well as Ab-mediated complement activation with subsequent uptake of Ag via complement receptors (29, 48, 51). Fc-independent mechanisms include Ab masking of dominant antigenic epitopes, exposure of cryptic epitopes revealed upon Ab binding, and/or changes in proteolysis that lead to changes in Ag presentation (49, 51–58). Results of the current study using \textit{Fcer1g} transgenic BALB/c mice demonstrated that beneficial immunomodulation by anti-P1 mAbs is independent of activating FcRs. Additional experiments substantiated that the primary mechanism of beneficial immunomodulation by anti-P1 mAbs 6-11A and 5-5D did not require the Fc region to mediate their effects. These results corroborate other studies in our laboratory that showed that beneficial immunomodulatory properties of anti-P1 mAbs do not partition with their ability to promote opsonophagocytosis or to activate complement (32).

Previously, an epitope-scanning approach evaluating T cell and linear B cell epitopes revealed that the response to P1 varied depending on the MHC II haplotype of the murine host (59). The effects of beneficial mAbs correspond with a shared feature of their epitopes, i.e., a common structural requirement for the interaction of the discontinuous A and P regions of P1. If, as suggested by changes in the specificity of the subsequent immune response, these mAbs act in some way as to perturb P1 structure and expose other epitopes, one would predict the immunodominant response against P1 to vary depending on the MHC II haplotype and genetic background of the host. An influence on P1 structure would be expected to lead to changes in Ag processing and presentation, the subset of stimulated Th cells, and the ultimate B cell response. The stimulation of particular T cell subsets is remarkably dependent on minor alterations in an Ag (52). The current study tested and confirmed the contribution of host genetic background in immunomodulation by anti-P1 mAbs.

The unifying property of anti-P1 mAbs that promote a desirable adherence-inhibiting Ab response is a common intramolecular interaction necessary for formation of their cognate epitopes, implying that the mechanism responsible for their redirection of the immune response involves the nature of the Ag-Ab interaction.

![FIGURE 7. Evaluation of immunogenic properties of recombinant P1 polypeptides. A. BIAcore SPR analysis of \textit{S. mutans} adherence to SAG. Sensograms show the binding of \textit{S. mutans} to immobilized SAG in the presence of sera collected from sham-immunized (PBS) BALB/c mice compared with those immunized with GC14 (full-length recombinant P1) or with truncated polypeptides NR21, CK1, and CK2. B. Sera collected from mice immunized with CG14 (•), NR21 (□), CK1 (△), CK2 (■), MBP alone (○), or acrylamide alone (△) were tested for their total IgG response against \textit{S. mutans} whole cells by ELISA. The percentage of inhibition of biotin-labeled 1-6F to \textit{S. mutans} whole cells by sera from mice immunized with CG14 (•), NR21 (□), CK1 (△), CK2 (■), MBP alone (○), or polyacrylamide alone (△) is shown by line graph. The SD between replicate samples was less than 5% in all cases.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/).
itself. Interestingly, binding of the anti-Ag I/II mAb Guy’s 13 reported to confer long-term protection as part of a passive immunization approach in human clinical trials also requires an A/P region interaction for formation of its cognate epitope (60–64). P1 binding by three of our four A/P-dependent mAbs (6-11A, 5-5D, and 3-10E) involves an additional contribution from pre-A region sequence (aa 84–190), and in-frame deletion of this region increases binding of the adherence-inhibiting mAb 1-6F to P1 even though mAb 1-6F maps to the intervening region between the A and P regions (aa 465–679) (38). Hence, it is tempting to speculate that binding of these immunomodulatory mAbs to P1 on the S. mutans cell surface has a similar effect on exposure of the 1-6F epitope. Collectively, our data indicate that binding of beneficial immunomodulatory anti-P1 mAbs to S. mutans increases the antigenicity and immunogenicity of a different epitope than their own. The most convincing evidence that immunomodulation is linked to epitope exposure and a structural perturbation of P1 on the cell surface is the demonstration that mAb 4-10A increases the binding of biotin-labeled mAb 1-6F to S. mutans whole cells at the same dilutions shown to promote mAb 1-6F-like responses and adherence inhibition activity in the serum of IC-immunized mice. Although the 1-6F epitope is not immunodominant in the absence of an uncomplexed beneficial mAb, it is detectable on S. mutans whole cells. Therefore, an increase in Abs against this epitope would be expected to function to block adhesion of colonizing bacteria in the oral cavity.

Further confirmation that structural modification of P1 alters its immunogenicity such that a more desirable response can be achieved was obtained in immunization experiments using the CK1 and CK2 derivatives of P1 that lack the post-P and pre-A region segments of the molecule. These regions were previously shown to contribute to P1’s native architecture on the cell surface (35). The comparable results obtained with the CK1 and CK2 constructs suggest that the pre-A region residues 84–185 are not critical to elicit an adherence-inhibiting response in the context of these two polypeptides. Even though the relevant 1-6F epitope is achieved in polypeptide NR21, the disparity between the results of adherence inhibition, whole-cell ELISA, and 1-6F competition experiments compared with CK1 and CK2 suggests that epitopes other than 1-6F may also be important targets of adherence-inhibiting Abs. In addition, the poor immunogenicity of NR21 compared with CK1 and CK2 may relate to a lack of Th cell epitopes. When RANKPEP was used to predict P1 peptides that would interact with highest affinity with class II MHC molecules of BALB/c mice, NR21 contained a single peptide predicted to be displayed by I-A^d, whereas CK1 and CK2 contained three of five and four of five of the I-A^d-predicted peptides and three of five and four of five of the I-E^d-predicted peptides, respectively.

The current study provides new information regarding a strategy to manipulate the immune response toward the induction of anti-P1 Abs with a more desirable characteristic, i.e., inhibition of S. mutans adherence to its known physiological receptor. In addition to a potential therapeutic modality, the insight gained regarding specific mAb-mediated changes in the elicited immune response suggests that immunization with immune complexes in an animal model can also be used as a screening tool to learn the qualities of a more useful immune response. The approach of Ab-mediated immunomodulation either as a direct therapy or as a guide to immunogen re-engineering and achievement of an effective immune response may be broadly applicable to numerous pathogens beyond S. mutans for which vaccines are not yet available and that share the common characteristic of being able to persist in the face of a suboptimal immune response.

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Disclosures
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Corrections


Fig. 2D was published incorrectly. Serum samples from three groups of mice were examined in multiple experiments and the data were illustrated in multiple histograms. In Figs. 3–6, the order of the groups from left to right was mice immunized with bacteria alone (white bars), mice immunized with bacteria coated with the high Ab concentration (black bars), and mice immunized with bacteria coated with the low Ab concentration (gray bars). The order of the groups is the same in Fig. 2D; however, the bacteria-only group (left) was mistakenly indicated by a black bar and the high Ab group (middle) was mistakenly indicated by a white bar. The correct Fig. 2 is shown below. The published legend is correct but is shown again for reference.

**FIGURE 2.** Host genetic background affects beneficial immunomodulation. *A*, BIAcore SPR analysis of *S. mutans* adherence to immobilized SAG. Sensograms show the binding of *S. mutans* to immobilized SAG in the presence of serum from BALB/c or C57BL/6 mice immunized with *S. mutans* alone or with IC containing mAbs 6-11A or 5-5D at high (saturating) or low (0.1× subsaturating) concentrations. *B*, Evaluation of IgG subclasses reactive with anti-*S. mutans* whole cells. Sera collected 7 days after primary immunization from BALB/c or C57BL/6 mice immunized with *S. mutans* alone (diamonds), IC containing mAb 6-11A at the higher concentration (squares), or lower concentration (triangles) were evaluated by quantitative ELISA. *C*, Same as *B*, except that mAb 5-5D was used in the experiment. *D*, A competition ELISA was used to determine the level of mAb 1-6F-like Abs in the serum of BALB/c and C57BL/6 mice immunized with ICs of mAb 6-11A and 5-5D compared with *S. mutans* alone. The percentage of inhibition of binding of biotin-labeled 1-6F to *S. mutans* by sera from mice immunized with bacteria alone (two black bars), high mAb concentration IC (one black bar), and low mAb concentration IC (gray bar) is indicated. Results are expressed as mean ± SEM, and statistical significance compared with serum from *S. mutans*-only immunized mice is indicated by *p* values. Data are representative of at least three independent experiments. Statistical analysis was performed using GraphPad Prism 4.0, and analysis included one-way ANOVA.

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