Monoclonal Antibody-Mediated Modulation of the Humoral Immune Response against Mucosally Applied Streptococcus mutans

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Systemic immunization with antigen coupled to monoclonal antibody (MAb) has been used by several investigators to increase the number of MAb-producing hybridomas against an antigen and to elicit antibodies specific for poorly immunogenic epitopes. This strategy has implications for vaccine design in that protective immunity is not necessarily directed at immunodominant epitopes of pathogens and may be improved by deliberately shifting the immune response toward subdominant epitopes. To our knowledge, no studies to date have addressed the potential for immunomodulatory activity mediated by MAb bound to mucosally applied antigen. To test whether administration of an exogenous MAb directed against a streptococcal surface protein could influence the humoral immune response, BALB/c mice were immunized orally by gastric intubation or intranasally with Streptococcus mutans alone or S. mutans complexed with a MAb directed against the major surface protein P1. Significant changes in the subclass distribution, as well as the specificity, of anti-P1 serum immunoglobulin G antibodies were demonstrated in groups of mice which received S. mutans coated with the anti-P1 MAb versus those which received S. mutans alone. Alterations in the humoral immune response were dependent on the amount of anti-P1 MAb used to coat the bacteria. In addition, differences in the anti-P1 immune responses were observed between groups of mice immunized via oral versus intranasal routes. In summary, an exogenous MAb complexed with a streptococcal antigen prior to mucosal immunization can influence the immunoglobulin isotype and specificity of the host humoral immune response against the antigen.

Binding of different monoclonal antibodies (MAbs) to a vaccine antigen prior to parenteral immunization has been reported to exert a variety of immunomodulatory effects, including suppression, enhancement, and differences in the specificity of the elicited response (4, 5, 6, 69, 80). Protection against colonization with any microorganism would be expected to depend on induction of antibodies of the correct specificity and isotype. Immunomodulation by MAb represents a strategy to enhance protective immunity of vaccine antigens by inducing the formation of antibodies against subdominant but protective epitopes, by suppressing the immune response against nonprotective epitopes, and/or by altering the subclass distribution of immunoglobulins to more effective isotypes (4, 45, 56, 86, 87).

Streptococcus mutans is a major etiologic agent of dental caries (18, 41). The M, ~185,000 surface protein of S. mutans serotype c is variously referred to as P1 (16), antigen I/II (62), antigen B (66), and Pac (52). P1 is a member of a family of structurally complex cell surface-anchored multifunctional adhesins originally identified as antigens I and II (62), with antigen II being a carboxy-terminal breakdown product of antigen I/II. As reviewed by Jenkinson and Demuth (30), antigen I/II-like polypeptides are produced by virtually all species of oral streptococci that are indigenous to the oral cavity. They are comprised of multiple ligand-binding sites. Discrete regions within these polypeptides are reported to bind human salivary glycoproteins, other microbial cells, calcium, collagen, laminin, keratin, and fibronectin.

The gene encoding P1, called spaP or pac, has been cloned (37, 52) and sequenced (31, 53) by two groups. Notable features of P1 include a 38-residue amino-terminal signal sequence, a series of three 82-residue alanine-rich repeats within the amino-terminal third of the molecule, a 150-residue variable region where sequence differences between P1 and Pac are clustered, a series of three 39-residue proline-rich repeats in the central portion of the molecule, and carboxy-terminal sequences characteristic of wall- and membrane-spanning domains of streptococcal surface proteins. A panel of 11 unique anti-P1 MAbs was generated previously (1). These have been evaluated for reactivity with a number of truncated P1 polypeptides to determine their approximate binding sites (7, 8). One of the anti-P1 MAbs, 6-11A, was tested in this study and found to exhibit immunomodulatory activity. MAb 6-11A binds to a complex determinant which is dependent on the presence of the central proline-rich region of P1, although it does not bind directly to the P region itself (7). The P region has been found to be important for the structural integrity of the molecule and is necessary for surface expression of P1 (7).

Data supporting a role for humoral immunity against human dental caries have been reported for many years. Animal studies of the induction of protective immunity against S. mutans colonization and formation of dental caries have focused primarily on two antigens, P1 and glucosyltransferase (18). Studies of P1 have evaluated the immunogenicity of the entire molecule or fragments of the antigen by using a variety of adjuvants and bacterial vector delivery systems, usually administered via a mucosal route (21–24, 28, 29, 60, 63–65, 67, 71, 77, 83, 84). To try to direct the immune response against regions of...
P1 believed to be involved in adherence to salivary components, immunization with A region or amino-terminal fragments of P1 have been undertaken but have not yet achieved the same level of protection as immunization with the full-length protein (22, 67, 71). Investigators have also attempted to elucidate protective humoral immune responses against P1 by studying naturally sensitized humans (32, 48). These studies utilized synthetic peptides and focused on short linear B-cell epitopes of P1. Kelly et al. (32) reported limited antibody responses against sequences identified by them as epitopes of P1 and results consistent with the success of S. sobrinus in colonizing the oral cavity. Few data are available regarding protective immunity directed against complex P1 epitopes; however, Kelly et al. (32) did observe a significantly higher proliferative response of lymphocytes isolated from low-caries individuals against a particular T-cell epitope. Brattalh et al. (10) also pointed out the complexity of the relationship between dental caries and immune specificity. Their results suggested that low-caries children mounted a more diverse salivary IgA response against sonicated antigens of S. mutans and S. sobrinus and reacted against determinants not recognized by high-caries children.

Taken together, studies of naturally sensitized humans suggest that subtle differences in immune responses among caries-resistant and caries-susceptible individuals may be crucial for protection. Such differences may not be readily apparent by measuring total antibody levels against S. mutans or P1. Immunomodulation by MAb represents a strategy by which the immunodominant epitopes of a mucosally administered vaccine antigen such as P1 can be shifted. Because of the interest in P1 as a potential vaccine antigen against human dental caries (reviewed in reference 20), this study was undertaken to evaluate whether an exogenous anti-P1 antibody coupled to S. mutans prior to mucosal immunization would be capable of influencing the host’s humoral immune response against the organism. Indeed, an anti-P1 MAb bound to the surface of S. mutans prior to mucosal immunization was found to alter the humoral immune response in mice both in terms of the distribution of anti-P1 serum immunoglobulin G (IgG) subclasses and the specificity of the anti-P1 serum IgG response. To the best of our knowledge no other studies have directly addressed the question of potential immunomodulatory activity mediated by a MAb complexed with antigen and delivered via a mucosal route. This approach will enable questions regarding the correlation between protection and specificity of the elicited response, as well as isotype distribution, to be addressed in the future.

**MATERIALS AND METHODS**

**Immunizations and sample collections.** S. mutans NG8 was kindly provided by K. W. Knox, Institute for Dental Research, Sydney, Australia. Live bacteria were used for immunizations. Groups of six female 26–30-day-old BALB/c mice (Charles River Laboratories, Wilmington, Mass.) were immunized orally by gastric intubation or intranasally with buffer only, with NG8 alone, with NG8 + 6-11A (high) alone, with NG8 + 6-11A (low) alone, or with 6-11A only, with NG8 coated with 10-fold-less 6-11A (1:1,000), or with 6-11A alone, with NG8 reacted with a saturating dilution (1:100) of anti-P1 MAb 6-11A, and with NG8 reacted with a saturating dilution (1:100) of anti-P1 MAb 6-11A, with NG8 coated with 10-fold-less 6-11A (1:1,000), or with 6-11A alone at the saturating dilution. The control groups which received 6-11A only were included to ensure that measured effects were the result of an interaction of the MAb with S. mutans and not a result of an idioype-anti-idioype phenomenon. MAb 6-11A maps to the central region of P1. It recognizes a conformational epitope and has been shown to be dependent on the presence of the central proline-rich tandem repeat region for reactivity (7). The immunogens and routes of inoculation are summarized in Table 1.

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**Preparation of bacterial suspensions.** S. mutans and S. sobrinus were harvested by centrifugation, washed in buffer, and resuspended to a concentration of 1.5 × 10⁹ CFU/ml. Sterile NaHCO₃ (0.1 M) was the buffer used for oral immunizations, and phosphate-buffered saline, pH 7.2 (PBS), was used for intranasal immunizations. The bacterial suspensions were mixed with an equal volume of 6-11A diluted as indicated per group in the appropriate buffer. The mixtures of bacteria and MAb were rotated end-over-end for 1 h at 37°C, washed twice with buffer, and resuspended to 1.5 × 10⁹ CFU/ml. Orally immunized mice received 1.5 × 10⁷ CFU NG8 suspended in 100 μl of NaHCO₃ by gastric intubation. Intranasally immunized mice received 1.5 × 10⁷ CFU suspended in 10 μl of PBS in each nostril. Immunizations were done on days 1, 3, 6, 8, 10, 12, 14, 33, 35, 37, and 40. Saliva, vaginal wash, and serum samples were collected prior to immunization.

**Saliva and sample collection.** Saliva samples were collected on day 45, and vaginal wash and serum samples were collected on days 52 to 54. Saliva samples (~200 μl) were collected after subcutaneous injection of 0.11 ml of sterile pilocarpine nitrate (1 mg/ml; Sigma, St. Louis, Mo.). Vaginal washes were collected by flushing the vagina with 100 μl of NaHCO₃. An equal volume of dithiothreitol (0.01 M) was added to the washes, and the samples were incubated on ice for 10 min to reduce viscosity. Serum samples were collected by anesthetizing the mice with sodium pentobarbital (70 mg/kg; Butler, Columbus, Ohio) intraperitoneally and bleeding them from the retroorbital sinus or by cardiac puncture and exanguination. All saliva, vaginal wash, and serum samples were stored at −20°C until assayed.

**Measurement of anti-S. mutans and anti-P1 responses by ELISA.** Mucosal and serum samples were assayed for antibody against S. mutans NG8 whole cells, purified P1, and the A and P region domains of P1 by enzyme-linked immunosorbent assay (ELISA). Costar High Binding plates (Corning Incorporated, Corning, N.Y.) were used. Sample wells were coated overnight at 4°C in a moist chamber with 100 μl of 0.1 M carbonate-bicarbonate buffer (pH 9.6) containing 0.02% sodium azide and approximately 10⁵ NG8 whole cells, 500 ng of P1, 100 ng of A region, or 100 ng of P region. Coating buffer and unbound antigens were removed from the ELISA plate wells, and unreacted sites were blocked by the addition of 300 μl of PBS containing 0.5% bovine serum albumin and 0.03% Tween 20 and overnight incubation at 4°C. Plates were washed four times with PBS containing 0.03% Tween 20 (PBS-Tw) prior to use. All washes and dilutions of primary and secondary antibodies were done by using PBS-Tw. Peroxidase-labeled goat anti-mouse IgG and IgA (Cappell) and peroxidase-labeled goat anti-rabbit IgG (Cappell) were used at a 1:1,000 dilution. All secondary antibody conjugates were affinity purified.

**TABLE 1. Summary of murine immunizations**

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the murine IgA myeloma MOPC 315 as the standard. Comparable total IgA levels were detected in all saliva samples and in all vaginal wash samples. All serum samples were tested in duplicate as described above for levels of anti-NG8 and anti-P1 IgG and anti-P1 IgA. sera were diluted 1:1,000 to assay for specific IgG and 1:100 to assay for specific IgA. sera were tested for levels of IgG reactive with the isolated A and P regions of P1 at a 1:1,000 dilution. To test for relative levels of IgG1, IgG2a, IgG2b, and IgG3 subclasses against P1, sera from groups 2, 3, and 4 were assayed at a 1:1,000 dilution and from groups 7, 8, and 9 at a 1:500 dilution. Rabbit anti-mouse IgG subclass specific reagents (Miles) were diluted 1:1,000. Binding of the unlabeled rabbit anti-mouse IgG subclass-specific reagents was traced by using peroxidase-labeled goat anti-rabbit IgG.

Statistical analyses. The nonparametric Kruskal-Wallis test for completely randomized design was used to detect shifts in the location of groups. Wilcoxon’s rank sum test was further used to compare separate pairs of populations in order to reveal which two populations differed significantly in location when the Kruskal-Wallis test showed statistically significant differences between the groups.

NCS digestion of P1. To assess changes in antibody specificity against P1, a spectrum of P1 polypeptides was generated by using chemical treatment with N-chlorosuccinimide (NCS) for Western immunoblot analysis. Surface proteins were extracted from S. mutans NG8 with 0.5 M phosphate buffer (pH 6.0) as previously described (8). One milliliter of phosphate extract (approximately 10 mg of total protein) was electrophoresed on 7.5% sodium dodecyl sulfate (SDS) preparatory slab gels (14 cm by 14 cm by 1.5 mm), the gels were stained with Coomassie brilliant blue R250, and the P1 bands were excised with a scalpel blade. Gel slices were stored at −20°C until experiments were performed. NCS digestion of P1 was performed and visualized in the gel slices. Gel slices were washed in water for 30 min at room temperature and then equilibrated in 25 ml of digestion buffer (1:1:1 [wt/vol/vol] urea-glacial acetic acid-water) for 1 h at room temperature with two changes. Gel slices were incubated in 25 ml of digestion buffer containing 50 mM NCS (Sigma) for 40 min at room temperature and transferred to 50 ml of SDS sample buffer (63 mM Tris-Cl, pH 6.8; 10% glycerol; 2% SDS; 0.006% bromphenol blue). The SDS sample buffer was changed every 10 to 15 min over the next 2 h until the pH of the gel slice had been neutralized, as indicated by the color of the bromphenol blue. Each gel slice was ground to a fine paste with a mortar and pestle and then suspended in 1 ml of SDS sample buffer.

Western immunoblot reactivity of murine sera against NCS-generated P1 polypeptides. The gel slurry containing NCS-digested P1 was applied to 10% SDS preparatory slab gels (8.5 cm by 7 cm by 1.5 mm). Prestained high-molecular-weight protein standards (Sigma) were run in the marker lane of each gel. After electrophoresis the digested P1 polypeptides present in the gel were electroblotted onto nitrocellulose (pore size, 0.45 mm; Schleicher and Schuell). The bands revealed after substrate development were scanned and integrated on the basis of optical densities by using a high-resolution camera connected to a densitometer and to a computer (Gel Doc 1000 system; Bio-Rad). The Multi Analyst software program (Bio-Rad) was used to manipulate the camera data. Integration was carried out under visual control, and each blot strip was scanned individually. A calibration curve was constructed by reference to the prestained high-molecular-weight protein standards included on each blot, allowing the determination of the molecular mass of the detected peaks. The antibody activity spectrum of each blot strip was represented as a curve of optical density values, (in arbitrary units) versus calibrated molecular mass (in kilodaltons). This method has been used previously to evaluate and document differences in the specificity of immunoglobulins in serum and mucosal secretions against complex mixtures of antigens (2). Mean curves representing the optical density values of the six mice in a given group were generated by using the Multi Analyst program, allowing the spectrum of the reactivity of the antibodies to be compared among the groups. Because of the potential for subtle variations in the efficiency of NCS digestion, in the migration of the P1 polypeptides, or in the efficiency of transfer to nitrocellulose, direct comparisons were made only between sera from groups of mice analyzed on the same Western blot. Three groups of six mice were analyzed on each blot. Four replicate blots, each probed with a different IgG subclass-specific reagent, were analyzed in each experiment.

RESULTS

Anti-S. mutans mucosal immune responses. All mice immunized either orally (groups 2, 3, and 4) or intranasally (groups 7, 8, and 9) with S. mutans or S. mutans coated with anti-P1 MAB 6-11A at either concentration developed measurable IgA against whole NG8 cells as determined by ELISA (Fig. 1). Anti-NG8 IgA was measurable in both saliva (panel A) and vaginal wash fluid (panel B), a finding indicative of a common mucosal immune response. The degree of reactivity did not appear to be influenced by precoating the cells with MAB 6-11A. Anti-NG8 responses were not detected in buffer-only control groups (1 and 6) or MAB-only control groups (5 and 10). All preimmune saliva, vaginal wash, and serum samples were shown by ELISA to be nonreactive with S. mutans antigens (data not shown). Because of the limited volumes of saliva and vaginal wash fluid, no further analyses of the anti-S. mutans mucosal responses were performed.

Anti-S. mutans and anti-P1 serum immunoglobulin responses. The development of serum responses against the immunizing S. mutans was expected based on previous reports of induction of systemic immunoglobulins against streptococcal antigens administered both orally (28) and intranasally (82). The evaluation of anti-NG8 and anti-P1 serum immunoglobulin responses by ELISA is shown in Fig. 2. Orally immunized mice (groups 2, 3, and 4) developed higher levels of anti-NG8 serum IgG than intranasally immunized mice (7, 8, and 9) (P < 0.02) (panel A). Anti-NG8 serum IgG was readily detectable in intranasally immunized mice when samples were analyzed at a 1:500 dilution (data not shown). Interestingly, animals in group 3, which received NG8 coated with a saturating concentration of MAB 6-11A, demonstrated decreased levels of anti-NG8
serum IgG compared with group 2 animals, which received NG8 alone \( (P < 0.02) \). There was no apparent effect of pre-coating NG8 with 6-11A on the level of anti-NG8 serum IgG in intranasally immunized mice.

The levels of serum IgG against purified P1 in groups 2, 3, and 4 and in groups 7, 8, and 9 were similar to those measured against NG8 whole cells (panel B). Decreased reactivity in sera from group 3 mice compared to group 2 mice \( (P < 0.02) \) was also observed against purified P1.

The levels of reactivity of serum IgA against purified P1 were also measured (panel C). In contrast to serum IgG, intranasal immunization was clearly more effective at eliciting anti-P1 serum IgA than was oral immunization \( (P < 0.02) \). There were no statistical differences in the levels of anti-P1 serum IgA among groups of mice which received NG8 alone versus NG8 coated with anti-P1 MAb 6-11A.

To test whether differences in the specificity of serum IgG antibodies against particular fragments of P1 could be detected in sera from mice immunized with NG8 with or without MAb 6-11A, ELISA assays were performed with the purified proline-rich and alanine-rich repeat regions of P1 (Fig. 3). Minimal reactivity was detected against the P region in the sera from animals in any of the groups (panel A). This is not surprising in light of previous results which demonstrated that while the P region is important for the structural integrity of conformational epitopes within the central portion of P1, this domain itself is not highly antigenic \( (7) \). On the other hand, sera from orally immunized mice which received NG8 alone (group 2) demonstrated readily measurable serum IgG responses against the purified A region polypeptide (panel B). Group 4 mice, which received NG8 coated with a 0.1× saturating concentration of MAb 6-11A demonstrated similar levels of anti-A region IgG as the group 2 mice; however, the reactivity of sera from group 3 animals, which received NG8 coated with a saturating concentration of 6-11A, was significantly decreased \( (P < 0.02) \). Therefore, much of the decrease in serum IgG reactivity against P1 in group 3 mice can be accounted for by a decrease in reactivity against the A region. Significantly less IgG reactive with the A region was also observed in the sera of all intranasally immunized mice (groups 7, 8, and 9) compared with orally immunized mice in group 2 \( (P < 0.02) \).

**Anti-P1 serum IgG subclass responses.** The relative levels of each serum IgG subclass reactive with P1 were also measured by ELISA in orally and intranasally immunized mice (Fig. 4). Oral immunization with NG8 alone elicited approximately equal levels of anti-P1 serum IgG1, IgG2a, and IgG2b (panel A). A low level of anti-P1 serum IgG3 was also detected. Coating of NG8 with a saturating concentration of 6-11A prior to oral immunization elicited a notably different distribution of anti-P1 IgG subclasses in the serum of group 3 compared to group 2 mice. The levels of anti-P1 IgG1, IgG2a, and IgG2b were all significantly reduced \( (P < 0.04, P < 0.02, \) and \( P < 0.02, \) respectively); however, as can be seen in Fig. 4A, the reduction in anti-P1 IgG1 was more moderate. Therefore, IgG1 became the most readily detectable subclass of anti-P1 IgG in the sera of group 3 animals. No alterations in IgG subclass responses against P1 were observed in group 4 compared to group 2 animals.

The distribution of anti-P1 serum IgG subclasses in intranasally immunized mice was different from that observed for orally immunized mice and was not affected by MAb 6-11A in the same way (Fig. 4B). Measurable levels of anti-P1 IgG2a and IgG2b were readily detected in the sera of intranasally

**FIG. 2.** Serum immunoglobulin reactivity against NG8 whole cells and purified P1 measured by ELISA. (A) Serum IgG reactivity against NG8 whole cells. (B) Serum IgG reactivity against P1. (C) Serum IgA reactivity against P1. Significantly higher levels of anti-NG8 and anti-P1 IgG were detected in sera from group 2, 3, and 4 mice compared to group 1 and 5 mice \( (P < 0.02) \). Significantly higher levels of IgA were detected in sera from group 7, 8, and 9 mice compared to group 6 and 10 mice \( (P < 0.02) \).

**FIG. 3.** Serum IgG reactivity against purified fragments of P1 measured by ELISA. (A) Serum IgG reactivity with the proline-rich repeat domain (P region). (B) Serum IgG reactivity with the alanine-rich repeat domain (A region).
immunized mice in groups 7, 8, and 9, but IgG1 was not a predominant subclass. Also in contrast to orally immunized mice, precoating NG8 with MAb 6-11A did not result in any significant change in the subclass distribution of anti-P1 serum IgG in intranasally immunized mice.

**Alterations in specificity of anti-P1 serum IgG subclass responses.** To assess changes in antibody specificity, overlapping P1 polypeptides were generated by partial cleavage of the protein with NCS. NCS cleaves proteins at tryptophan residues. There are eight tryptophan residues within P1 at positions 582, 594, 643, 746, 759, 768, 811, and 1183 of the predicted amino acid sequence (31). A partial digestion of P1 is predicted to yield a cluster of three carboxy-terminal fragments of \( M_r \approx 100,775 \) to 107,679, a second cluster of four carboxy-terminal fragments of \( M_r \approx 82,147 \) to 89,450, and a cluster of seven amino-terminal fragments of \( M_r \approx 58,647 \) to 84,109, as well as 30 additional cleavage products ranging from \( M_r \approx 1,034 \) to 124,775. By utilizing MAbs that bind to the A region and carboxy terminus of P1, the presumptive identity of certain clusters of bands was established.

A summary of the Western blot experiments using sera from orally immunized mice is illustrated in Fig. 5. Sera from individual animals were analyzed for reactivity against NCS-digested P1 on four replicate Western blots with rabbit anti-

![Fig. 4](image_url)

FIG. 4. Serum IgG subclass reactivity against purified P1 measured by ELISA. (A) Orally immunized mice: □, group 2; ▣, group 3; ■, group 4. (B) Intranasally immunized mice: □, group 7; ▣, group 8; ■, group 9.

![Fig. 5](image_url)

FIG. 5. Binding of serum IgG subclasses from orally immunized mice to NCS generated P1 polypeptides, as demonstrated by computer-assisted by Western blot analysis. The curves represent the mean densitometry profiles of the six mice in each group. The ordinate corresponds to the intensity of bands detected by the antibodies, and the abscissa indicates the apparent molecular masses (in kilodaltons) of the corresponding antigens, as calculated from the electrophoretic migration reciprocal distances. Lines: ——, group 2; ——, group 3; ——, group 4.
mouse IgG subclass-specific reagents as probes. Each curve represents the mean densitometry profile for the six animals in the indicated group. The optical density (in arbitrary units) is shown on the y axis. The calibrated molecular mass is represented on the x axis. Three major groups of reactive bands were observed on Western immunoblot strips, depicted in the graphs as peaks. The ~120-kDa peak corresponds to a cluster of bands reactive with the carboxy-terminal-specific MAb 3-3B (8). The ~100-kDa peak also likely represents a cluster of carboxy-terminal fragments based on the predicted size. The cluster of bands corresponding to the ~85-kDa peak was reactive with the A region-specific MAb 3-8D (15).

As expected from ELISA experiments, there was an obvious decrease in the level of anti-P1 serum IgG from group 3 mice. This was observed for all four subclasses. There was a predominant IgG1 response in both group 2 and 4 animals, followed by an IgG2b response, against epitopes present in NCS-digested P1 fragments. IgG2a and IgG3 responses were also measurable. Although no difference in the levels of anti-P1 serum IgG was detected by ELISA between group 2 mice (NG8 alone) and group 4 mice (NG8 coated with a 0.1× saturating concentration of MAb 6-11A), a striking difference in the specificity of the responses was observed by Western blot. Sera from group 2 mice were more reactive with the ~85-kDa peak corresponding to amino-terminal P1 fragments. Sera from mice in group 4 demonstrated a marked shift away from recognition of amino-terminal fragments with increased reactivity directed against the ~120-kDa cluster of carboxy-terminal P1 fragments. Minimal antibody reactivity of any subclass was detected against the ~100-kDa cluster of bands, and this reactivity was not substantially different between group 2 and group 4 mice.

Sera from intranasally immunized mice were also analyzed by Western blot (Fig. 6). Based on the ELISA reactivity, serum samples from intranasally immunized mice were tested at a 1:100 dilution rather than the 1:1,000 dilution used for orally immunized mice. IgG2a and IgG2b were the predominant anti-P1 subclasses detected by ELISA in sera from groups 7, 8, and 9; however, IgG1 and IgG3 antibodies reactive with NCS-digested P1 were also detected by Western blot. In contrast to orally immunized mice, sera from intranasally immunized mice demonstrated a broader spectrum of reactivity against partially digested P1. Additional cleavage products were recognized by antibodies present in the sera of intranasally immunized mice. The peak indicated by the asterisk in Fig. 6 corresponds to Mr ~185,000 and represents a small amount of undigested P1. A lower degree of digestion could have contributed to the change in densitometry profiles observed between Fig. 5 and 6. However, control Western blot strips reacted with MAbs 3-8D, 3-3B, and polyclonal rabbit antiserum appeared to be identical in both experiments, suggesting that this was not the major reason for the difference. The change more likely represents differences in antibody specificity resulting from oral versus intranasal immunization. Control blot strips probed with an anti-P1 polyclonal rabbit antiserum (data not shown) demonstrated numerous small P1 fragments on all blots that were not reactive with sera from either orally or intranasally immunized mice. This suggests that mucosal immunization with whole S. mutans cells is not effective at generating serum IgG antibodies reactive with small linear segments of P1.

Mean densitometry profiles were nearly identical for mice from groups 7, 8, and 9 when Western blot strips were probed with individual murine sera followed by IgG1, IgG2b, and IgG3 murine subclass specific reagents (Fig. 6). However, precooling

![Graphs showing IgG subclass reactivity](https://via.placeholder.com/150)
NG8 with a 0.1× saturating concentration of MAb 6-11A did result in a detectable alteration in the specificity of the serum IgG2a response against NCS-digested P1. The notable increase in reactivity of group 9 compared to group 7 and 8 mice corresponds with the largest cluster of NCS digestion products recognized by anti-P1 MAb 3-3B. Similar to the shift in specificity of orally immunized mice in group 4, the change in specificity of group 9 mice most likely represents an increase in reactivity against the ~120-kDa cluster of carboxy-terminal polypeptides.

Taken together, the results outlined above demonstrate that the spectrum of P1 epitopes recognized by oral versus intranasal immunization with whole NG8 cells is not identical. The relative levels of anti-P1 serum IgG and IgA, as well as the subclass distribution of serum IgG, also vary depending on the route. Anti-P1 MAb 6-11A demonstrates immunomodulatory activity when bound to S. mutans administered by both routes, but the effects are not entirely the same. A saturating concentration of 6-11A bound to S. mutans prior to oral immunization resulted in an alteration of the relative levels of anti-P1 serum IgG subclasses, as well as a demonstrable shift in the specificity of serum IgG, particularly notable for IgG1 and IgG2b subclasses, away from amino-terminal toward carboxy-terminal P1 fragments. A 0.1× saturating concentration of 6-11A bound to S. mutans prior to intranasal immunization affected the specificity of the IgG2a response, also apparently increasing recognition of large carboxy-terminal fragments of P1.

**DISCUSSION**

The potential to modulate an immune response by systemic immunization with antigen bound by antibody has been recognized for some time (11, 26, 33, 36, 47, 54, 68, 75, 78, 79). More recent work has begun to elucidate some of the underlying molecular mechanisms by which antibody may alter an immune response. Antibody may influence antigen uptake by antigen-presenting cells (46). In addition, certain MAbs complexed with a protein antigen have been shown to alter proteolytic processing of the antigen by antigen-presenting cells (45). This in turn would be expected to modulate the fine specificity of the B-cell response (37). As stated earlier, Kelly et al. (32) indicated that subtle differences in the T-cell response against P1 could be detected between caries-resistant and -susceptible individuals. In a study designed to analyze the effect of immunization with antigen-antibody complexes on the repertoire of antigen-reactive B cells at the molecular level, Nie et al. (51) examined the rearranged immunoglobulin heavy-chain variable (V_H) genes from mouse splenic germinal centers. These authors reported that mice immunized with an antigen-MAb complex demonstrated more-heterogeneous V_H gene expression than mice immunized with antigen alone. Bratthall et al. (10) have reported that low-caries children demonstrate a more diverse anti-S. mutans response than high-caries children. A strategy which could increase or shift the spectrum of epitopes recognized during an anti-S. mutans response would therefore be expected to be of therapeutic benefit.

The fact that S. mutans can colonize and persist in humans in the face of measurable antibody responses suggests that naturally immunodominant epitopes may not be optimal for protection. Both anti-P1 sIgA in saliva and anti-P1 serum IgG, which would enter the oral cavity via crevicular fluid, have been reported to confer protection against colonization by S. mutans and dental caries (13, 39, 40, 61, 74). However, the specificity and subclass of highly protective antibodies have not been thoroughly defined. This study was undertaken to ascertain whether an anti-P1 MAb bound to the surface of S. mutans could be used to redirect the predominant immune response against the organism. Many mucosal immunization protocols have the advantage of eliciting both secretory IgA as well as systemic IgG responses (28, 49, 64, 85). Two commonly used routes of mucosal immunization, gastric intubation and intranasal administration, were chosen because these routes have been used successfully to induce both mucosal and serum immunoglobulin responses against streptococcal surface proteins (28, 82). Both routes were studied because of the potential that the specificity of antibodies induced against S. mutans antigens might differ depending on the route of immunization (17, 76). Indeed, class and subclass distribution and specificity of anti-P1 antibodies in the sera of orally and intranasally immunized animals were found to differ and were affected differently by the anti-P1 MAb.

All animals which received S. mutans NG8, with or without MAb, by either route developed measurable anti-NG8 sIgA in both saliva and vaginal fluid, a result indicative of a common mucosal immune response. The degree of sIgA reactivity did not appear to be affected by precoating the bacterial cells with 6-11A. Because of the limited volumes of mucosal samples, extensive analyses of the specificity of the sIgA response could not be made. Experiments to assess changes in the specificity of the elicited humoral response were performed with serum samples where sample volumes were not limiting. Orally immunized mice developed higher titers of anti-P1 serum IgG than intranasally immunized mice, while intranasally immunized mice responded with higher levels of anti-P1 serum IgA. Differential expression of tissue-specific adhesion molecules has been demonstrated on circulating antibody-forming cells after systemic, enteric, and nasal immunizations (56, 57); therefore, the route would be expected to play a role in the isotype distribution of systemic immunoglobulins elicited by mucosally administered antigens. Orally immunized mice also exhibited a different profile of anti-P1 serum IgG subclasses than intranasally immunized animals. Anti-P1 IgG1, IgG2a, and IgG2b were all measurable by ELISA in the sera of orally immunized mice, whereas intranasally immunized mice demonstrated predominantly anti-P1 IgG2a and IgG2b. Orally immunized mice which received NG8 coated with a saturating concentration of MAb 6-11A demonstrated significantly lower levels of anti-P1 serum IgG, with anti-P1 IgG2a and IgG2b levels reduced to a greater extent than IgG1. No obvious change in the anti-P1 serum IgG subclass distribution was observed in orally immunized mice which received NG8 coated with a 0.1× saturating concentration of 6-11A, nor in intranasally immunized mice which received NG8 coated with MAb at either concentration. These results indicate that the immunomodulatory effect of an antibody can vary depending on its concentration as well as on the route of immunization.

Information regarding an alteration in subclass response is particularly interesting since the isotype of an antibody has been shown to be as important as the specificity with regard to its ability to mediate protection. Yuan et al. have demonstrated in a mouse model of Cryptococcus neoformans infection that deliberately engineering an isotype switch from an IgG3 MAb to an IgG1 MAb of identical specificity converted a nonprotective murine MAb to a protective one (86, 87). To date, no studies have defined an optimally protective anti-P1 serum IgG subclass. The ability to direct the anti-S. mutans response toward a particular subclass by employing a combination of appropriate route and immunomodulatory MAb used at an appropriate concentration will enable experiments to be designed to answer this question.
Both ELISA and Western blot experiments demonstrated that the specificity of anti-P1 serum IgG antibodies were altered when mice were mucosally immunized with S. mutans coated with MAb 6-11A. Again, changes were dependent on both the route of immunization and the coating concentration of the immunomodulatory MAb. As stated above, orally immunized mice which received S. mutans saturated with MAb 6-11A demonstrated lower total levels of anti-S. mutans and anti-P1 serum IgG than animals which received bacteria alone or bacteria coated with a 0.1× saturating concentration of MAb. Binding of different MAbs to a vaccine antigen prior to parenteral immunization has been reported to exert both suppressive and enhancing effects on the magnitude of the elicited response (5, 69, 80). In this instance, much of the decrease in anti-P1 serum IgG reactivity could be accounted for by a decrease in reactivity against the A region as measured by ELISA. This result is interesting in that MAb 6-11A has been mapped to the central region of P1 and does not react at all with the A region (7, 8). The immunogenicity of the A region was clearly altered by a MAb whose specificity is directed against a different region of the protein. This result is not surprising in light of previous studies by Bougie et al. (5) and Coulie and Snick (14), who showed changes in the immunogenicity of domains of antigens nonreactive with immunomodulatory MAbs. A shift in recognition away from amino-terminal determinants was also observed when Western blot experiments were performed with P1 that had been partially digested with NCS. Mice that had been immunized either orally or intranasally with S. mutans coated with MAb 6-11A demonstrated increases in reactivity against large carboxy-terminal fragments of P1. Changes in the specificity of anti-P1 IgG1, IgG2a, and IgG2b were observed in orally immunized mice, whereas the increased recognition of an Mr ~120,000 carboxy-terminal P1 polypeptide was confined to the IgG2b subclass in intranasally immunized mice.

The potential clinical relevance of the observed change in specificity of the anti-P1 serum IgG response is difficult to predict and will be the focus of future studies. Published studies indicate that immunization with amino-terminal fragments of P1 elicited partial protection (22, 67, 71), while the results of Munro et al. (50) and Kelly et al. (32) suggest that determinants carboxy terminal to the P region are most important. Partial digestion of P1 with NCS has proven to be a most valuable tool in the evaluation of changes in specificity of anti-P1 responses. A spectrum of overlapping polypeptides is generated by this method so that subtle differences in specificity can be identified that might be obscured merely by comparing antibody titers against the full-length molecule. Previous studies have utilized overlapping synthetic peptides to evaluate humoral responses against P1 (32, 48), but such studies detect antibodies directed against small linear epitopes only. As demonstrated in this study, responses against more complex epitopes can be evaluated by using limited proteolytic breakdown products of P1. Brady et al. (9) and Hajishengallis et al. (19) have reported that ligand binding by P1 is most likely mediated by conformational determinants; therefore, effective protection by antibody would be expected to involve recognition of complex epitopes.

Consistent with our findings of immunomodulatory activity associated with an anti-S. mutans MAb, several investigators have suggested that an exogenously administered antibody may act as a therapeutic agent by redirecting the host response against an infectious agent rather than playing a purely passive role (5, 27, 55, 58, 59, 72, 86). Bougie et al. showed that parenteral immunomodulation by MAb could occur with different types of antigens, including a human soluble protein, specifically slgA, a bacterial polysaccharide from E. coli K1, and an envelope protein from the hepatitis B virus (5, 6). Generation of antibodies recognizing novel epitopes by immunization with immune complexes or sequential administration of specific antibody followed by antigen has been achieved for feline CD4 (81) and Actinobacillus pleuropneumoniae (70) and has been used to elicit protective immunity against Newcastle disease virus in chickens (55).

There is a growing interest in the use of mucosally applied antibodies as therapeutic agents (3, 12, 25, 34, 35, 43, 44), including protection against human dental caries. Local oral passive immunization with an anti-P1 MAb (Guy’s 13) has been reported to prevent recolonization of human volunteers with S. mutans following treatment with chlorhexidine (42–44). Treated individuals were reported to remain free of S. mutans for up to 2 years after a 3-week treatment period. Guy’s 13 is similar to MAb 6-11A in that it binds to a determinant within the central region of P1 (50). In light of results demonstrated here, it is possible that the mechanism of protection in the treated patients was one of immunomodulation and enhancement of protection mediated by an immune complex consisting of the passively applied MAb and low levels of recolonizing S. mutans. The possibility that passively administered antibody used therapeutically to treat a streptococcal infection may work via an immunomodulatory mechanism has already been suggested by Ramisse et al. (58). Human plasma-derived immunoglobulin (IVIG) administered either intravenously or intranasally within 2 h of challenge with Streptococcus pneumoniae was protective in a murine model against pneumococcal pneumonia. Mice protected with IVIG developed higher levels of measurable antibodies against pneumolysin and acquired greater resistance to subsequent reinfection than untreated survivors, strongly suggesting that exogenous anti-streptococcal antibodies can potentiate the development of protective adaptive immunity.

In summary, the results described here indicate that an MAb directed against a streptococcal surface protein bound to the bacterial surface prior to mucosal immunization can influence the subclass distribution and specificity of elicited antibodies. Either of these changes has the potential to alter the protective capacity of the humoral immune response. The evaluation of additional anti-P1 MAbs and the effect of observed changes in the immune response with respect to protection against S. mutans colonization and cariogenicity will be the focus of future work. Information regarding mucosal immunomodulation mediated by exogenous antibody would be relevant to the study of any active or passive mucosal immunization approach.

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REFERENCES


