INFECTION AND IMMUNITY, Jan. 2004, p. 13–21 Vol. 72, No. 1
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.1.13–21.2004

Further Characterization of Immunomodulation by a Monoclonal Antibody against *Streptococcus mutans* Antigen P1

Nikki R. Rhodin, Marloes L. J. A. Van Tilburg, Monika W. Oli, William P. McArthur, and L. Jeannine Brady*

Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, Florida 32610-0424

Received 24 February 2003/Returned for modification 1 July 2003/Accepted 25 September 2003

We demonstrated previously that mucosal immunization of mice with *Streptococcus mutans* coated with the monoclonal antibody (MAb) 6-11A directed against the major surface adhesin protein P1 results in changes in the amount, isotype distribution, and specificity of serum antibodies compared with animals immunized with bacteria only. We now show that the specificity of the mucosal secretory IgA response was also influenced by this MAb. Changes in antibody specificity were associated with changes in biological activity. Serum samples which differed in antibody reactivity with P1 polypeptides generated by partial digestion with *N*-chlorosuccinimide but not in isotype distribution or overall reactivity with *S. mutans* or intact P1 demonstrated a statistically significant difference in the ability to inhibit bacterial adherence to salivary-agglutinin-coated hydroxyapatite beads. Serum IgG antibodies against P1 from mice immunized with either *S. mutans* alone or *S. mutans* coated with 6-11A were shown to recognize antigenic determinants dependent on the presence of the central proline-rich repeat domain, a segment necessary for the structural integrity of the molecule. However, no statistically significant differences were observed in antibody reactivity with a panel of six partial P1 polypeptides encoded by overlapping *spaP* subclones, suggesting that the targets of biologically relevant antibodies involve complex epitopes not reconstituted by the recombinant products tested. Lastly, we show that binding of MAb 6-11A to P1 on the surface of *S. mutans* alters P1's susceptibility to proteolytic digestion. Hence, changes in antigen processing and presentation may contribute to the immunomodulatory effects of this MAb.

Antibodies of appropriate specificity and isotype are important for an optimal protective humoral immune response against a pathogen. Immunomodulation by exogenous antibodies, in which the antigen is complexed with antibody prior to immunization, can be used to deliberately shift reactivity away from immunodominant but nonprotective epitopes towards subdominant but more protective epitopes (3, 32). We have identified an immunomodulatory monoclonal antibody (MAb) that recognizes the P1 surface adhesin of *Streptococcus mutans*. The immunogenicity of mucosally applied *S. mutans* in BALB/c mice is altered when MAb 6-11A is complexed with P1 on the cell surface (8).

S. mutans is the etiologic agent of human dental caries and both secretory immunoglobulin A (sIgA) and serum IgG antibodies have been reported to contribute to protection (36). Parenteral immunization with *S. mutans* whole cells can prevent colonization and development of caries in nonhuman primates (4, 29). More recent studies have focused on defined antigens, including P1, a member of the antigen I/II family of surface adhesins found on many oral streptococci. Studies evaluating P1's immunogenicity have utilized the entire molecule or fragments thereof and a variety of adjuvants and bacterial vector delivery systems, usually mucosally administered. Many mucosal immunization protocols have the advantage of eliciting both sIgA and serum IgG responses (35).

S. mutans possesses several virulence factors that enable it to colonize and dominate its niche in the oral cavity. The ~185,000-M_r wall-associated fibrillar lectin, identified as antigen I/II (46) and known as P1 (15), Pac (43), antigen B (47), IF (20), or MSL-1 (14), is widely presumed to promote attachment of *S. mutans* to the acquired pellicle on teeth via specific binding to a high-molecular-weight glycoprotein called salivary agglutinin (22). The gene encoding P1, called *spaP* or *pac*, has been cloned (28, 42) and sequenced (24, 43). P1 and antigen I/II homologues exhibit an amino-terminal signal sequence, a series of amino-terminal alanine-rich repeats (A region), a series of central proline-rich repeats (P region), and carboxy-terminal sequences characteristic of wall and membrane-spanning domains, including a second proline-rich region, an LPXTG motif, and a charged cytoplasmic tail.

Immunomodulatory MAb 6-11A is reactive with *S. mutans* whole cells (6) and recognizes a complex determinant of P1 dependent on the presence of the proline-rich repeat domain of P1, while not binding to the P region directly (5). Immunomodulatory effects of 6-11A vary depending on the route of mucosal immunization and on the coating concentration of the antibody (8). Binding of MAb 6-11A to *S. mutans* prior to immunization of mice by gastric intubation influences the isotype distribution of the anti-P1 serum IgG response and the specificity of serum IgG antibodies against large polypeptide fragments of P1 generated by partial digestion with *N*-chlorosuccinimide (NCS). Serum antibodies from animals immunized with *S. mutans* alone are more reactive with large ~85-kDa amino-terminal fragments, whereas antibodies from mice immunized with *S. mutans* coated with a 0.1× subsaturating concentration of MAb 6-11A are more reactive with large ~120-kDa carboxy-terminal fragments.

The present study was undertaken to define further the

* Corresponding author. Mailing address: Department of Oral Biology, University of Florida, Gainesville, FL 32610-0424. Phone: (352) 846-0785. Fax: (352) 392-7357. E-mail: jbrady@dental.ufl.edu.

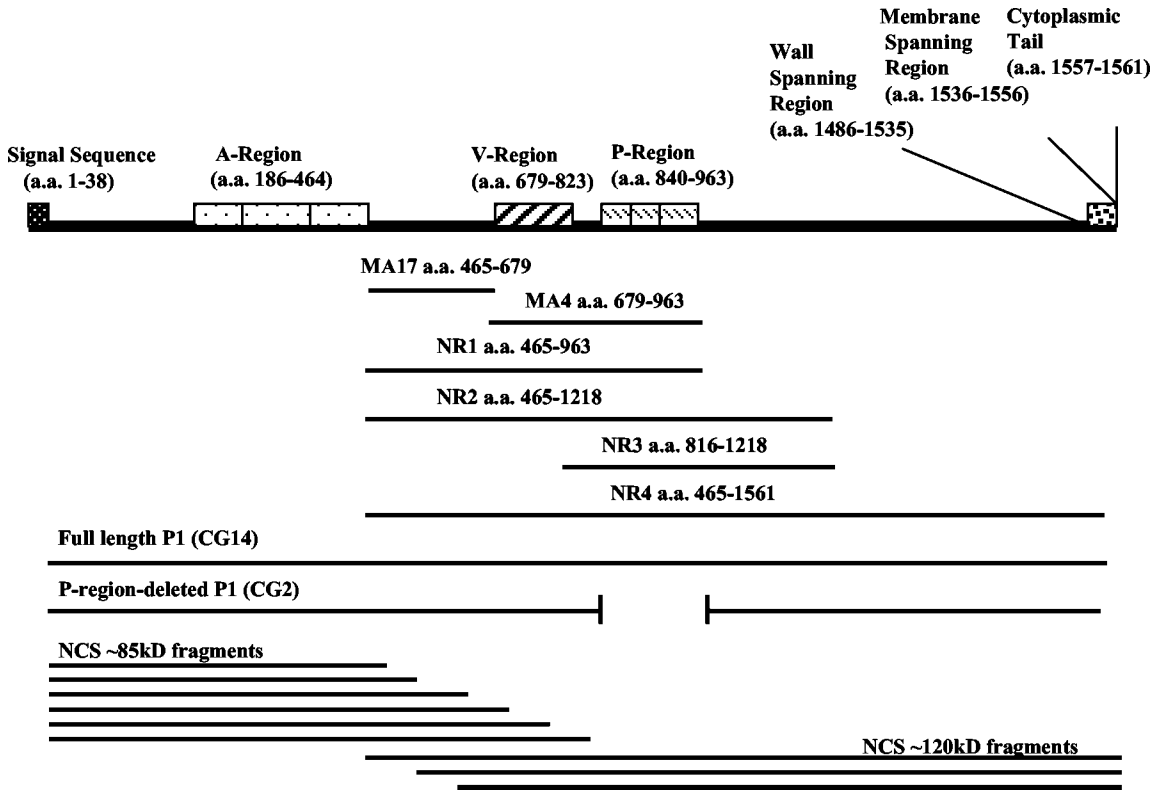


FIG. 1. Schematic of P1, *spa*P subclones, and predicted NCS cleavage products.

specificity and magnitude of serum IgG and mucosal sIgA antibody responses against P1, to evaluate whether changes in the antibody response are associated with changes in biological activity, and to begin to characterize a potential mechanism of action of immunomodulation by MAb 6-11A.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Serotype c *S. mutans* NG8 (kindly provided by K. W. Knox, Institute for Dental Research, Sydney, Australia) was grown aerobically to stationary phase for 16 h in Todd-Hewitt broth (BBL, Cockeysville, Md.) supplemented with 0.3% yeast extract. *Escherichia coli* host strains included DH5α, INVαF′ (InVitrogen Corp., San Diego, Calif.), and M15 (pREP4) (Qiagen, Santa Clarita, Calif.). *E. coli* was grown aerobically at 37°C with vigorous shaking in Luria-Bertani broth (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl) supplemented with ampicillin (50 to 100 μg/ml) or kanamycin (25 to 50 μg/ml). Plasmids pCR2.1 (InVitrogen Corp.), pQE30 (Qiagen), and pMal-p (New England Biolabs, Inc. [NEB], Beverly, Mass.) were used as cloning and expression vectors.

Anti-P1 monoclonal and polyclonal antibodies. Immunological reagents included murine MAb 6-11A (1) and two rabbit polyclonal antisera (5, 7). MAb 6-11A IgG1 was affinity purified from murine ascites fluid using a protein A cartridge and the BioLogic HR Workstation (Bio-Rad, Hercules, Calif.), dialyzed against phosphate-buffered saline (PBS) (pH 7.2) containing 0.3% sodium azide, aliquoted, and stored at −20°C. Antiserum 209 was made against P1 isolated by ion-exchange and gel filtration chromatography (7). Antiserum 218 was made against *S. mutans* NG8 whole cells (5) and rendered monospecific for P1 by exhaustive adsorption with the *spa*P-negative mutant PC3370 (11). Antisera 209 and 218 recognize overlapping but not identical epitopes of P1.

Murine immunizations and sample collections. Groups of six BALB/c mice (Charles River Laboratories, Wilmington, Mass.) were previously immunized by gastric intubation (8) with $NaHCO_3$ (0.1 M) buffer alone (group 1), *S. mutans* strain NG8 alone (group 2), NG8 coated with a 1× saturating concentration of anti-P1 MAb 6-11A (NG8[High 6-11A]) (group 3), or NG8 coated with a 0.1× subsaturating concentration of MAb 6-11A (NG8[Low 6-11A]) (group 4). The amount of MAb necessary to saturate the bacteria was determined by serial titration and dot blot analysis (8). Control immunizations included MAb 6-11A only and *S. mutans* mixed with an irrelevant IgG1 isotype-matched control MAb. No anti-P1 or NG8 reactivity was detected in these groups (8), and they were not analyzed here. Sera and vaginal wash fluids were stored at 20°C.

NCS digestion of P1 and Western immunoblot reactivity of sIgA. P1 was partially digested with NCS, and the products were electrophoresed on SDS-polyacrylamide gels and electroblotted onto nitrocellulose filters (8). Replicate Western blot strips were reacted with vaginal wash fluids, each diluted 1:20 in phosphate-buffered saline (PBS) containing 0.03% Tween (PBS-Tw). After washing, strips were reacted with biotin-labeled goat anti-mouse IgA (Southern Biotechnology, Birmingham, Ala.) diluted 1:250. Strips were washed again and reacted with extravidin (Sigma, St. Louis, Mo.) diluted 1:1,000, washed, and developed with 4-chloro-1-naphthol solution (7 ml of PBS, 1 ml of 4-chloro-1-naphthol [3 mg/ml in ice-cold methanol; Sigma], and 8 μl of 30% hydrogen peroxide). Control strips were reacted with appropriate anti-P1 MAbs.

Bands revealed after substrate development were scanned and integrated based on optical density using a high-resolution camera connected to a densitometer and computer (Gel Doc 1000 system; Bio-Rad) (8). The Multi Analyst software program (Bio-Rad) was used to manipulate the camera data. Mean curves representing the optical density values (in arbitrary units) for each group were generated.

Inhibition of *S. mutans* adherence to salivary agglutinin-coated hydroxyapatite. Inhibition of adherence of *S. mutans* whole cells to salivary-agglutinin-coated hydroxyapatite beads was assayed as previously described (7). Adherence inhibition was carried out using a 1:100 dilution of pooled sera from each treatment group. Adherence inhibition was determined for immune sera, with percent inhibition of preimmune sera subtracted as the background. Percent inhibition of adherence was calculated as the following: [(% adherence without sera − % adherence with sera)/% adherence without sera] × 100. Assays were performed in triplicate. Differences in percent inhibition were detected using Student's *t* test.

PCR and construction of *spa*P subclones. Sequences of interest within *spa*P (Fig. 1) were amplified by PCR using NG8 chromosomal DNA as the template with primers designed based on the published *spaP* sequence (28). Forward and reverse primers used to amplify MA17 (amino acids [aa] 465 to 679) were

5′-GGGGAGTACTGATTTAGACAGACTATCCA-3′ and 5′-GGGTCGACTCAGTCAGTCAGTCATAGAAAAGTGAAATC-3′, respectively; forward and reverse primers for MA4 (aa 679 to 963) were 5′-GGGAGTACTGACGAAGATGGAAAACCA-3′ and 5′-GGGTCGACTCAGTCA-GTCACTCATAAGTTGGCTCAAC-3′, respectively. NR1 (aa 465 to 963) was engineered using the forward primer for MA17 and reverse primer for MA4. NR2 (aa 465 to 1218) was amplified using the forward primer designed for MA17 and 5′-GGGTCGACTCAGTCAGTCAATCCTGACGCAATTCAAG-3′ as the reverse primer. NR3 (aa 816 to 1218) was amplified using forward primer 5′-GGAGTACTGGTAAAATCCGTGCGGTTAA-3′ and the reverse primer engineered for NR2. NR4 (aa 465 to 1561) was amplified using the forward primer for MA17 and the reverse primer 5′-GGGTCGACTCAGTCAGTCAATCTTTCTTAGCCTTTAA-3′. Underlined sequences in forward primers correspond to a *Sca*I recognition sequence. Underlined sequences in reverse primers correspond to a *Sal*I recognition sequence. Restriction sites were engineered for cloning into pMal-p (NEB). Translational termination codons in all three reading frames were included in reverse primers to prevent read-through into the β-galactosidase gene of pMAL-p.

PCR was carried out for 30 cycles as follows: (i) denaturation at 94°C, 1 min; (ii) primer annealing 51°C, 2 min; (iii) primer extension 72°C, 2 min. Primer extension was carried out for an additional 5 min following the last cycle. Amplified PCR products were cloned into pCR2.1 (Invitrogen Corp) according to the manufacturer's instructions. Recombinant plasmids were restricted with *Sca*I (Promega, Madison, Wis.) and *Sal*I (Promega), and insert DNA was ligated into *Stu*I (Promega)- and *Sal*I (Promega)-linearized pMal-p (NEB). Sequences of all recombinant constructs were confirmed by the DNA sequencing core (University of Florida).

Purification of P1-MBP fusion proteins. P1–maltose-binding protein (MBP) fusion proteins were purified by amylose resin chromatography (NEB) from periplasmic or cytoplasmic preparations of *E. coli* DH5α harboring recombinant pMal-p according to the manufacturer's instructions. Recovery of P1-MBP fusion proteins was confirmed by Western immunoblotting using anti-P1 polyclonal antisera and anti-MBP polyclonal antibody (NEB). Purified fusion proteins were quantified using the bicinchoninic acid protein assay kit (Sigma) with bovine serum albumin as the standard.

Measurement of murine antibodies by ELISA. Serum samples were assayed for anti-P1 IgG antibodies by enzyme-linked immunosorbent assay (ELISA) as previously described (8). Approximately 100 ng of each protein per well was used for coating. Murine sera were diluted 1:225 to measure reactivity against the MA4 and MA17 fusion proteins and were diluted 1:5,000 for all others. All serum samples were tested in duplicate. Antibody reactivity was traced using affinity-purified peroxidase-labeled goat anti-mouse IgG (ICN/Cappell ICN Biomedicals, Aurora, Ohio) diluted 1:1,000. Successful coating of recombinant proteins was confirmed using anti-P1 and/or anti-MBP polyclonal rabbit antisera and affinity-purified peroxidase-labeled goat anti-rabbit IgG (ICN/Cappell). 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 revealed statistically significant differences between the groups.

Isolation of full-length recombinant P1 and P1ΔP. Recombinant full-length P1 and P1 with the P region deleted (P1ΔP) were engineered as previously described (5). *E. coli* M15 harboring the pQE30 vector (Qiagen), pCG14 encoding full-length P1, or pCG2 encoding P1ΔP were induced with 1 mM isopropyl-β-D-thiogalactopyranoside, harvested, and lysed according to the manufacturer's suggested protocol. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 7.5% gels and stained with Coomassie brilliant blue G (Sigma). Bands corresponding to full-length P1 and P1ΔP were excised, and gel slices were minced with a razor blade. Proteins were eluted with 50 mM ammonium bicarbonate buffer (pH 7.8) containing 0.1% SDS overnight at 37°C with gentle rocking. Recombinant proteins were dialyzed into water, and ELISA assays were performed as described above. Similar levels of coating of the two antigens was confirmed with anti-P1 MAb 3-8D, whose binding is not affected by removal of the P region (5). Differences in serum IgG reactivity between the treatment groups were detected using Wicoxon's rank sum test, and differences in reactivity between full-length P1 and P1ΔP within a given group were detected using Student's *t* test.

Proteolytic treatment of *S. mutans*. *S. mutans* bacterial cells coated with MAb 6-11A at a 0.1× subsaturating concentration (8) or control cells without antibody were treated with protease, and the products of digestion were analyzed by Western immunoblotting. The concentration of MAb 6-11A was chosen because it altered the specificity of the anti-P1 response in previous murine immunization experiments (8). A 0.1× subsaturating concentration was achieved with a 1:500 dilution of affinity-purified MAb 6-11A IgG (1.72 mg/ml in PBS).

NG8 cells were harvested from 50 ml of culture by centrifugation for 15 min at 2,000 × *g* (4°C) and washed twice with PBS, pH 7.2. For samples coated with MAb 6-11A, cell pellets were resuspended in 1 ml of PBS, mixed with an equal volume of PBS containing purified MAb 6-11A diluted 1:500, and rotated end over end for 1 h at room temperature. Bacteria were harvested by centrifugation and washed twice with PBS to remove unbound antibody. Washed bacteria with and without antibody were suspended in 1 ml of appropriate digestion buffer containing the indicated protease.

Proteases included sequencing-grade *Staphylococcus aureus* V8 protease (Roche Diagnostics Corp. Indianapolis, Ind.), sequencing-grade chymotrypsin (Roche), and endoproteinase Arg-C (Sigma). Digestion conditions were as follows. Bacterial cells with and without MAb were incubated with either 5 μg of V8 protease in 1 ml of 25 mM $(NH_4)_2CO_3$ (pH 7.8) at 25°C, 5 μg of chymotrypsin in 1 ml of 100 mM Tris-HCl, 10 mM $CaCl_2$ (pH 7.8) at 37°C, or 50 μg of endoproteinase Arg-C in 1 ml of 100 mM Tris-HCl–10 mM $CaCl_2$ (pH 7.6) at 37°C. Incubations ranged from 0 to 60 h depending on the protease. At each time point, a 200-μl aliquot of the cell suspension was removed, bacteria were pelleted at high speed for 5 min in a tabletop microfuge, and the supernatant was removed and boiled for 5 min in SDS sample buffer. Cell pellets were also resuspended and boiled for 5 min in 200 μl of SDS sample buffer. Samples were stored at −20°C until analyzed by SDS-PAGE and were boiled again prior to loading.

Controls included analysis of the zero time point and MAb 6-11A incubated with each protease in the absence of bacterial cells.

SDS-polyacrylamide gel electrophoresis and Western immunoblot analysis of P1 proteolysis. Products of digestion were separated by electrophoresis on either 10% or 7.5% polyacrylamide slab gels and electroblotted onto nitrocellulose filters for 1 h at 100 V. Replicate filters were stained with colloidal gold (Diversified Biotech, Boston, Mass.) or blocked for 1 h in PBS-Tw and then incubated overnight at room temperature with anti-P1 polyclonal antiserum 209 or 218 diluted 1:1,000 in PBS-Tw. Blots were washed, reacted with affinity-purified peroxidase-labeled goat anti-rabbit conjugate (ICN/Cappell) at a 1:5,000 dilution for 3 h, and developed with chemiluminescence substrate according to the manufacture's directions (ECL detection reagents, Amersham Biosciences). Control blots were reacted with affinity-purified peroxidase-labeled goat anti-mouse conjugate (ICN/Cappell) at a 1:5,000 dilution to identify bands corresponding to MAb 6-11A (data not shown). These were easily differentiated from P1 bands reactive with rabbit antisera and a rabbit-specific secondary reagent.

RESULTS

Alterations in the specificity of the anti-P1 sIgA response. Previously we described changes in the specificity of the serum IgG antibody response between mice immunized with *S. mutans* coated with a 0.1× saturating concentration of 6-11A and mice immunized with *S. mutans* alone (8). To determine if there were also changes in the specificity of the mucosal immune response, anti-P1 sIgA antibody in vaginal fluids from the same mice was evaluated by Western immunoblot analysis of NCS-generated P1 polypeptides.

The mean densitometry profiles for the six animals in each indicated treatment group are shown in Fig. 2. Optical density (in arbitrary units) is shown on the *y* axis, and the calibrated molecular mass is represented on the x axis. sIgA present in vaginal fluids from mice immunized with NG8[Low 6-11A] showed increased reactivity against the ~120-kDa cluster of bands, while sIgA present in vaginal fluids from mice immunized with NG8[High 6-11A] was more reactive with the ~85-kDa cluster of bands. Little sIgA reactivity against any NCS-generated P1 polypeptides was detected in animals immunized with NG8 alone. These results indicate that the intensity and specificity of the sIgA response is influenced by immunomodulatory MAb 6-11A and that such changes are dependent on the coating concentration of the antibody. Because of the limited

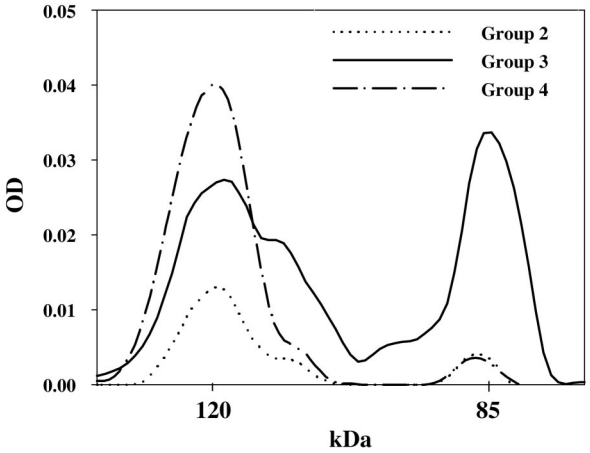


FIG. 2. Binding of sIgA from vaginal wash fluids from immunized mice to NCS-generated P1 polypeptides, as demonstrated by computer-assisted Western blot analysis. Each curve represents the mean densitometry profiles of the six mice in the designated treatment group. The ordinate corresponds to the intensity of bands detected by antibodies, and the abscissa indicates the apparent molecular masses (in kilodaltons) of the corresponding antigens. Group 2, NG8 alone; group 3, NG8[High MAb]; group 4, NG8[Low MAb].

volumes of vaginal wash fluids, all further experiments utilized serum samples.

Inhibition of *S. mutans* adherence to salivary agglutinin-coated hydroxyapatite beads. In order to determine if MAb 6-11A-mediated changes in the specificity of serum antibodies elicited following oral immunization are associated with changes in biological activity, an adherence inhibition assay was performed. As shown in Fig. 3, sera from mice immunized either with NG8 alone or with NG8[High 6-11A] demonstrated <10% inhibition of bacterial adherence to salivary agglutinin-coated hydroxyapatite beads. However, sera from mice immunized with NG8[Low 6-11A] demonstrated a statistically significant increase in inhibition (~25%) over that for the NG8 alone group ($P < 0.02$, Student's t test).

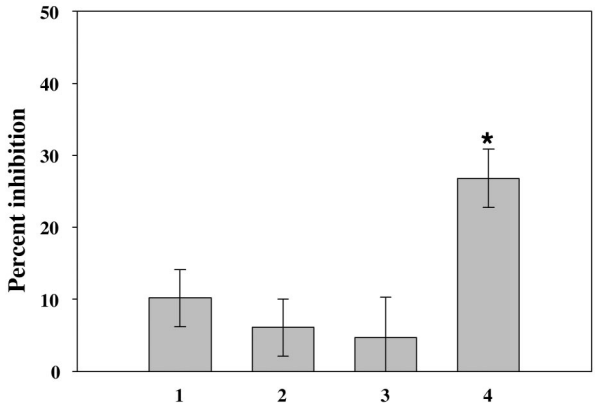


FIG. 3. Percent inhibition by serum antibodies of adherence of *S. mutans* to salivary-agglutinin-coated hydroxyapatite beads. The asterisk indicates significantly higher percent inhibition than with group 2 ($P < 0.02$). Group 1, buffer-only control; group 2, NG8 alone; group 3, NG8[High MAb]; group 4, NG8[Low MAb].

Evaluation of anti-P1 serum IgG antibody reactivity with defined recombinant P1 polypeptides. To test for other potential differences in the specificity of the anti-P1 serum IgG response, the reactivity of serum IgG antibodies with purified polypeptide products of subclones of *spa*P (Fig. 1) was evaluated by ELISA. Minimal binding to two polypeptides, MA4 corresponding to aa 465 to 679 and MA17 (aa 679 to 963) was detected by ELISA in any of the groups of immunized mice (Fig. 4). Because important conformationally dependent epitopes might be lost in smaller polypeptides, such as MA4 and MA17, larger polypeptide fragments spanning the central and carboxy-terminal region of P1 were generated and analyzed as well. Sera from mice immunized with NG8 alone had higher IgG antibody reactivity against NR1 (aa 465 to 963) ($P < 0.0082$), NR2 (aa 465 to 1218) ($P < 0.0082$), NR3 (aa 816 to 1218) ($P < 0.0202$), and NR4 (aa 465 to 1561) ($P < 0.0082$) than buffer-only control mice. Further, when serum IgG reactivity to NR4 was assessed, there was significantly less antibody detected in serum from NG8[High 6-11A]-immunized mice than in that from mice immunized with NG8 alone ($P < 0.0081$) or NG8[Low 6-11A] ($P < 0.0081$) animals. There were no significant differences in serum IgG antibody reactivity with any of the recombinant P1 polypeptides between those animals which received NG8 alone and those that received NG8[Low 6-11A] despite the notable difference in both serum IgG and sIgA reactivity against NCS-digested P1 and the statistically significant difference in serum inhibition of *S. mutans* adherence.

Murine serum IgG antibody reactivity with full-length P1 compared to that with P1ΔP. The reactivity of serum IgG antibody against purified full-length recombinant P1 and P1ΔP was measured by ELISA to determine if the P region contributes to epitopes differentially recognized by serum antibodies in the various treatment groups. The results of these experiments are shown in Fig. 5. Serum IgG antibody reactivity against full-length P1 was significantly increased in mice immunized with NG8 alone and NG8[Low 6-11A] compared to the buffer control group ($P < 0.01$). Serum IgG antibody reactivity against P1ΔP was significantly less for each treatment group than that for full-length P1 (NG8 alone [$P = 0.0218$], NG8[High 6-11A] [$P = 0.0078$], NG8[Low 6-11A] [$P = 0.0005$]), suggesting that the P region is necessary for the formation of epitopes recognized by anti-P1 serum IgG antibodies produced by mice in all three groups.

Influence of MAb 6-11A on susceptibility of P1 to proteolysis. In vitro susceptibility to proteolysis has been used as a tool to assess alterations in protein conformation (19, 23, 54) and to study antigen processing (2, 30, 53). To begin to explore whether changes in antigen processing might represent a potential mechanism of immunomodulation by MAb 6-11A, the susceptibility of cell surface P1 to digestion with a variety of proteases, including V8 protease, chymotrypsin, and endoproteinase Arg-C, was investigated. These proteases were chosen because of their broad range of predicted cleavage sites within P1. Samples derived from a V8 digest of MAb-coated (0.1× subsaturating concentration) and uncoated cells, as well as corresponding supernatants from the reaction tubes, were electrophoresed on SDS-polyacrylamide slab gels, transferred to nitrocellulose, and stained with colloidal gold (Fig. 6A and B). No notable difference in the overall pattern of proteolysis

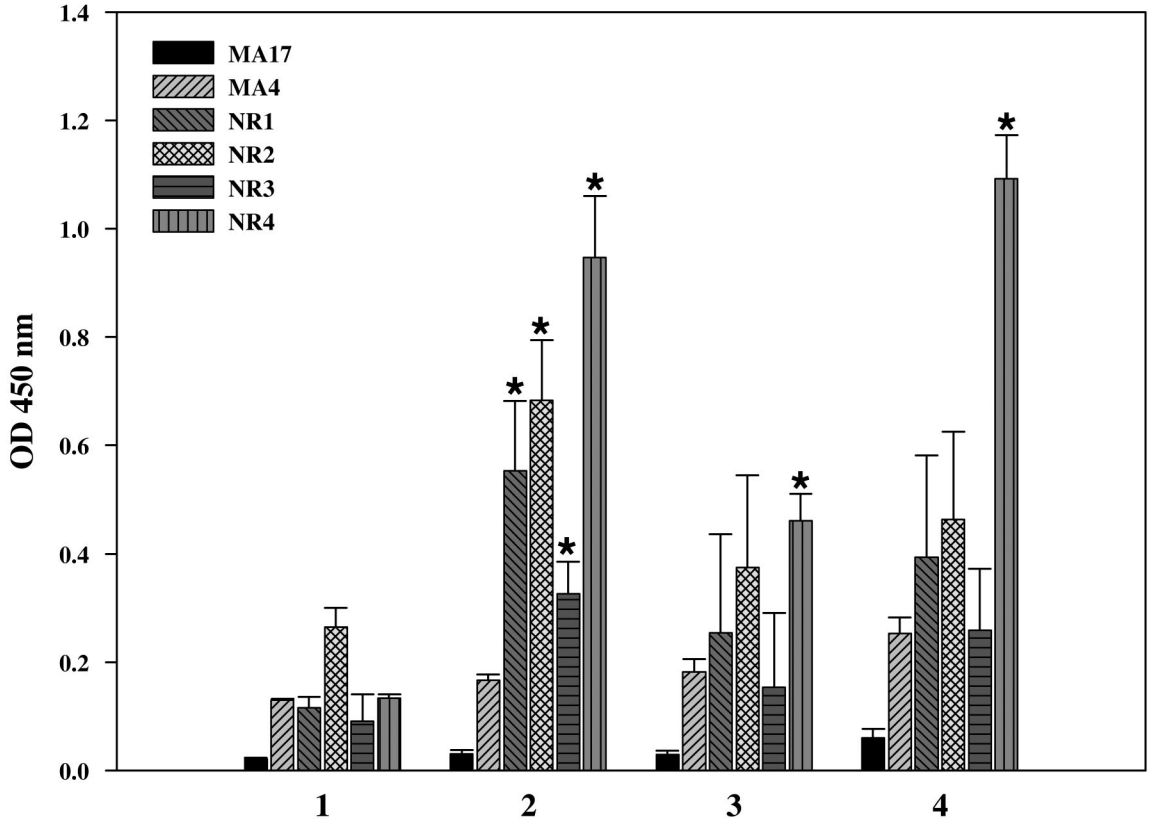


FIG. 4. Serum IgG antibody reactivity with purified P1 polypeptide fragments measured by ELISA. An asterisk indicates significantly higher levels of serum IgG detected than with group 1 ($P < 0.01$). Group 1, buffer-only control; group 2, NG8 alone; group 3, NG8[High MAb]; group 4, NG8[Low MAb].

in the presence or absence of MAb 6-11A at any time point was apparent irrespective of whether cells themselves or supernatants were analyzed. This was true for all of the proteases utilized in these experiments (data not shown).

To evaluate changes in the susceptibility of P1 itself to proteolysis in the presence and absence of MAb 6-11A, Western blots were reacted with the two different anti-P1 polyclonal antisera described in Materials and Methods. Western blots of MAb-coated and uncoated cells that were reacted with anti-P1 antisera 209 show that P1 is cleaved into smaller-molecular-weight fragments more rapidly in the presence of MAb 6-11A (Fig. 6C). This is most apparent at the 36-h time point, at which full-length P1 is no longer demonstrable. This same effect was also observed when the corresponding supernatants were analyzed (Fig. 6D). Further, at the 36-h and 48-h time points, particular P1 fragments were absent in the MAb 6-11A-treated sample that were apparent when uncoated cells were analyzed (Fig. 6C). Corresponding supernatants reacted with the same antiserum showed similar results (Fig. 6D).

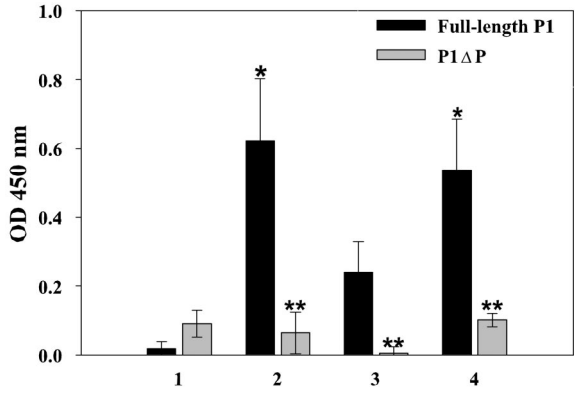
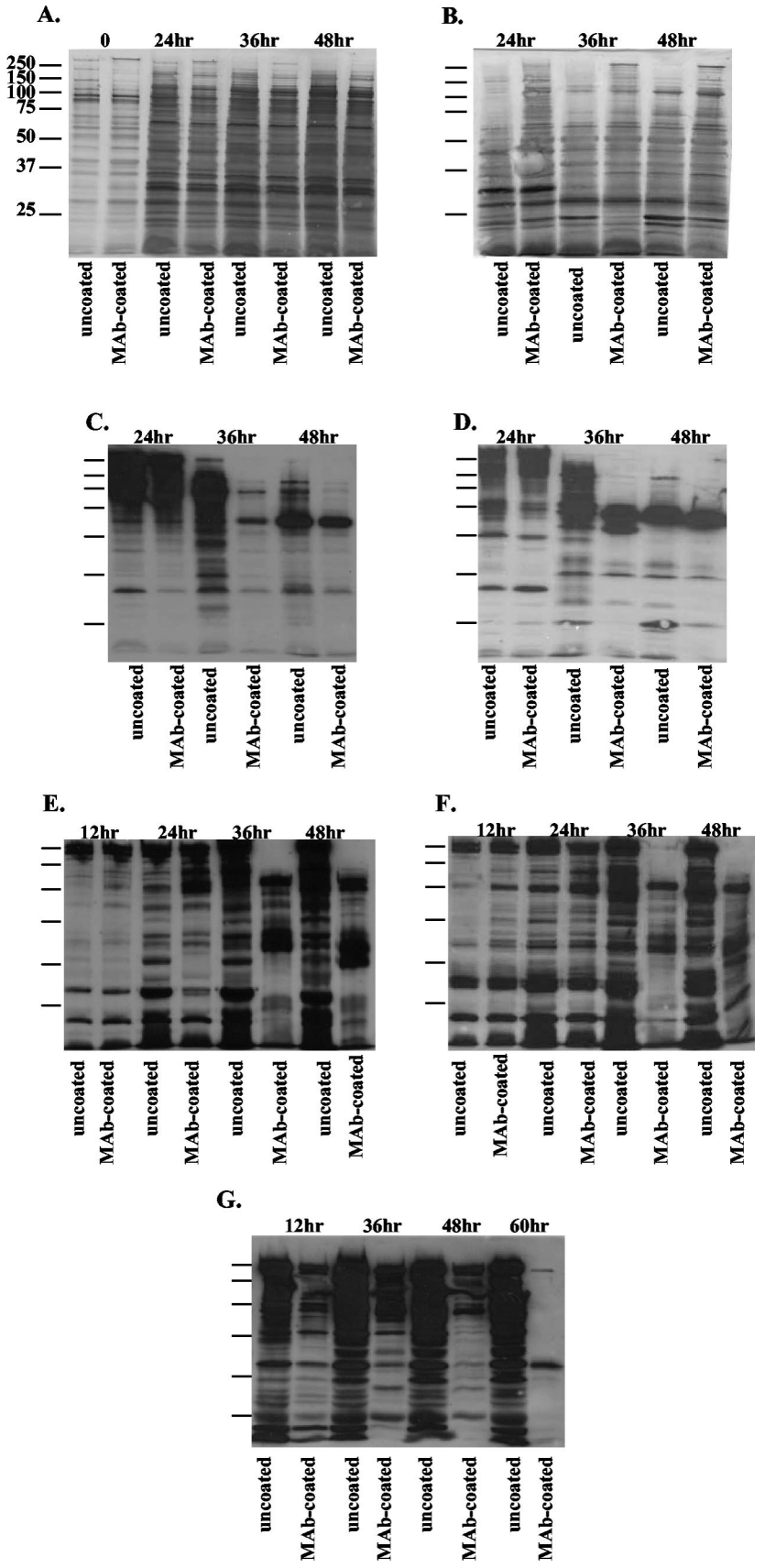


FIG. 5. Serum IgG antibody reactivity with recombinant full-length P1 and P1ΔP as measured by ELISA. A single asterisk indicates higher serum IgG reactivity than with group 1 ($P < 0.01$). Two asterisks indicate lower serum IgG reactivity than with full-length P1 in the same treatment group: for group 2, $P = 0.0218$; for group 3, $P = 0.0078$; for group 4, $P = 0.0005$. Group 1, buffer only control; group 2, NG8 alone; group 3, NG8[High MAb]; group 4, NG8[Low MAb].

To see if changes in susceptibility of P1 to proteolysis were unique to V8 protease, MAb-coated and uncoated *S. mutans* cells were also treated with chymotrypsin and endoproteinase Arg-C. Products of a chymotrypsin digestion present in reaction supernatants are shown in Fig. 6E (antiserum 209) and F (antiserum 218). Again P1 was digested more rapidly and more completely when MAb 6-11A was bound to P1 on *S. mutans* cells. In the absence of MAb 6-11A, intact P1 could be detected throughout the entire time course of the experiments. In addition, both antiserum 209 (Fig. 6E) and 218 (Fig. 6F) reveal the appearance of an additional band in the supernatant fraction of MAb 6-11A-coated *S. mutans* cells at the 48-h time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point that is not present in the absence of the MAb. Figure 6G shows the Western blot of reaction supernatants from an endoproteinase Arg-C digest of MAb 6-11A-coated and uncoated *S. mutans* NG8 cells reacted with antiserum 218. Again, the presence of 6-11A appears to promote the breakdown of P1 to smaller-molecular-weight products more rapidly than in its absence.

DISCUSSION

The ability to modulate an immune response by parenteral or mucosal immunization with an antibody bound to an antigen has been demonstrated (18, 34, 45, 51). Although factors which dictate whether an antibody complexed with an antigen will change an immune response are not fully understood, several mechanisms have been suggested, and they include increased uptake of antigen via Fc receptors on antigen-presenting cells (30), masking of dominant epitopes by antibody (2, 31), exposure of cryptic epitopes induced by antibody binding (49, 53), enhanced germinal center formation and induction of somatic hypermutation (26, 41), and/or alterations in proteolysis and antigen processing (30, 32, 49). Monoclonal antibody 6-11A against *S. mutans* adhesin P1 influences the specificity and isotype distribution of the serum IgG response in mice immunized with whole cells by gastric intubation (8).

In this study, experiments were designed to evaluate the specificity of sIgA antibodies following gastric immunization with *S. mutans* and to continue to search for potential changes in serum IgG reactivity mediated by MAb 6-11A. An in vitro adherence assay was used to evaluate biological activity of serum antibodies from mice that received *S. mutans* alone versus 6-11A-coated bacteria. A P1 polypeptide devoid of the P region, a domain necessary for the structural integrity of the molecule (5), was used to determine whether the antibodies were directed against conformational, possibly discontinuous, epitopes. We demonstrated that this was the case irrespective of the presence or absence of MAb 6-11A during immunization. Last, we observed that P1 on the surface of *S. mutans* was degraded more rapidly and completely by multiple proteases in the presence of MAb 6-11A. Patterns of digestion were also altered, suggesting that P1 may be cleaved at different target sites when bound by this MAb. Our data suggest that immunomodulation by MAb 6-11A may involve changes in antigen processing, although it does not preclude contributions by other mechanisms that have not yet been explored.

Previously, changes were detected in the specificity of serum IgG antibodies against P1 partially digested with NCS, even when no quantitative differences against intact P1 were apparent (8). Here we used the same approach to demonstrate an alteration in the specificity of the mucosal immune response. sIgA antibodies in vaginal fluids of mice immunized with NG8[Low 6-11A] demonstrated increased recognition of a cluster of ~120-kDa NCS-generated P1 fragments. Mice immunized with NG8[High 6-11A] showed increased sIgA reactivity against a cluster of ~85-kDa P1 fragments. Previous results with serum IgG from the same animals (8) had demonstrated strong reactivity with the ~85-kDa bands in the NG8-alone group and little reactivity with any of the NCS-generated P1 polypeptides in the NG8[High 6-11A] group. ELISA measurement of specific anti-*S. mutans* sIgA/total sIgA in vaginal wash fluids (data not shown) demonstrated that unlike the serum IgG response, the sIgA response was not diminished in the NG8[High 6-11A] animals compared to results with the other treatment groups. Taken together, our results indicate that the specificity of the sIgA response is altered by MAb 6-11A. Also, there is a difference in the immunomodulatory effect of MAb 6-11A on the serum IgG response compared to the sIgA response.

The potential relevance of observed changes in the specificity of anti-P1 antibodies was analyzed using an in vitro adherence inhibition assay (10). Sera from mice immunized with NG8[Low 6-11A] were more inhibitory of *S. mutans* adherence to hydroxyapatite beads than sera from mice immunized with NG8 alone ($P < 0.02$). This suggests that changes in the specificity of the anti-P1 response are associated with changes in biological activity.

Several studies suggest that the interaction of the antigen I/II family of molecules with salivary glycoproteins is complex and involves multiple binding sites (22). The ability of the isolated amino terminus of P1 to interact with salivary components has been reported (12, 37, 39, 43). Hajishengallis (16) has suggested that multiple sites within the amino-terminal two-thirds of P1 mediate adhesion. Adhesin activity has also been localized to amino acid residues 816 to 1213 (38), with residues 1005 to 1024 believed to play a key role (25). Analysis of antibody reactivity against NCS-generated P1 polypeptides (Fig. 2 and reference 8) and adherence inhibition assays (Fig. 3) suggest that antibodies against complex epitopes involving central and carboxy-terminal sequences can inhibit the interaction of P1 with salivary agglutinin. It is curious that sera from mice immunized with *S. mutans* alone, known to include antibodies that recognize the amino-terminal region of P1 (8), did not inhibit adherence of *S. mutans* to hydroxyapatite beads despite reports regarding the importance of antibodies against this segment (17). The most likely explanation for this disparity is that antibodies detected in all the studies can interact with large amino-terminal polypeptides, but the immunization procedures differ enough that identical epitopes are not recognized.

Because of differences in reactivity against NCS-generated fragments of P1, we wished to define further those regions of

FIG. 6. Effect of MAb 6-11A binding to *S. mutans* on susceptibility of cell surface P1 to protease digestion. For colloidal gold stain of products of V8 protease treatment of *S. mutans* with and without bound MAb 6-11A, polypeptides associated with cell pellets (A) and polypeptides released into the supernatant (B) are shown. For Western blot of products of V8 protease treatment of *S. mutans* with and without bound MAb 6-11A traced with anti-P1 antiserum 209, polypeptides associated with cell pellets (C) and polypeptides released into the supernatant (D) are shown. For Western blot of polypeptide products released into the supernatant following chymotrypsin treatment of *S. mutans* with and without bound MAb 6-11A, anti-P1 antiserum 209 (E) and anti-P1 antiserum 218 (F) are shown. For Western blot of polypeptide products released into the supernatant following endoproteinase Arg-C treatment of *S. mutans* with and without bound MAb 6-11A, anti-P1 antiserum 218 (G) is shown.

P1 recognized by antibodies from each treatment group. Polypeptide products of a panel of overlapping *spa*P subclones were used as antigens in ELISA assays (Fig. 1 and 4). Subclone NR4 in particular was constructed in an attempt to replicate epitopes contained within the ~120-kDa NCS-generated fragments differentially recognized by serum IgG and sIgA antibodies. There were no statistical differences in the levels of serum IgG antibody reactivity against any of the subcloned products between mice which received bacteria alone and those which received bacteria coated with MAb at either concentration. This suggests that epitopes differentially recognized by antibodies from the different groups of mice are complex and not reconstituted by the recombinant P1 polypeptides tested, yet these epitopes are maintained when full-length P1 is partially digested with NCS. Our results were similar whether we used native or recombinant NCS-digested P1 (data not shown), suggesting that posttranslational modifications are not at play in this system. Van Dolleweerd et al. (52) reported that a complex epitope results from interaction of the discontinuous A and P regions of P1; therefore, recombinant polypeptides which lack either or both of these domains would not achieve a native structure, and antibodies against higher-order structures would be nonreactive.

The P region is an important structural component of P1. Its presence is necessary for surface expression of P1 on *S. mutans* and is necessary, but not sufficient, for binding of five different anti-P1 MAbs, including immunomodulatory 6-11A (5). Several, but not all, of these MAbs inhibit in vitro adherence to salivary agglutinin-coated hydroxyapatite beads (7). Serum IgG reactivity with P1ΔP was decreased significantly for each treatment group compared to that with full-length P1, suggesting that the P region is necessary for the formation of epitopes recognized by antibodies elicited following gastric immunization of mice with *S. mutans* alone or MAb-coated bacteria. Our prior work has shown that serum antibodies from animals in each group are nonreactive with the isolated P region (8). Therefore, the lack of binding of serum IgG antibodies to P1ΔP indicates that immunization with bacteria, or MAb-coated bacteria, elicits antibodies specific for complex epitopes dependent on the presence of the central proline-rich region repeats.

Proteolysis of antigen-antibody complexes has been used to identify functional epitopes within an antigen. Because regions of an antigen in contact with an antibody are resistant to proteolysis (44), a bound MAb can protect against cleavage at specific target sites (21, 48). Resistance or susceptibility to proteolysis can also be used to indicate changes in protein conformation (19, 23, 54). In general, destabilization of structure is associated with exposure of cryptic target sites and faster and more extensive digestion. In addition to being used as tools in protein structure-function studies, proteases are integral components of the immune response itself. Native and destabilized proteins vary with regard to their immunogenicity (9, 13, 40, 50). Increases in structural flexibility and susceptibility to proteolysis are associated with a stronger and broader helper-T-cell response (9, 13, 50).

The observation that P1 was degraded more rapidly and completely by proteases in the presence of MAb 6-11A in vitro strongly suggests that binding of this MAb alters the protein structure of P1. Patterns of proteolysis of non-P1 proteins were not changed. With multiple proteases and two different anti-P1 antisera, we observed examples in which particular bands were more apparent when MAb 6-11A was present during the digestion phase and examples when the converse was true. This suggests that 6-11A may not only block particular cleavage sites, but additional cleavage sites might be targeted in the presence of the MAb. Our results indicate that MAb 6-11A influences proteolysis of cell-associated P1 in vitro and suggest that its immunomodulatory effects may result, at least in part, from changes in antigen processing in vivo.

Investigators have shown that presentation of particular antigen-specific T-cell determinants can be enhanced or suppressed as a direct consequence of antibody modulation of antigen processing (32, 49, 53). The prediction that changes in the specificity of T-cell epitopes would modulate the fine specificity of an antibody response (27) has been born out in studies of conformational epitopes of *E. coli* β-galactosidase (33). We speculate that changes in the susceptibility of P1 to proteolysis, as a result of binding of MAb 6-11A, could ultimately influence the spectrum of antibodies elicited during a polyclonal response. Such antibodies would include not only those recognizing linear epitopes but also more complex conformation-dependent determinants, some of which may be more effective inhibitors of adherence than others. Immunomodulation by monoclonal antibody therefore represents a promising approach to improve protection of a humoral immune response against a pathogen.

ACKNOWLEDGMENTS

We thank Beatrice Correa for her technical assistance.

This work was supported by NIH grant DE13882 and training grant T32-DE07200.

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Editor: T. R. Kozel