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Influence of Intranasal Immunization with Synthetic Peptides Corresponding to Conserved Epitopes of M Protein on Mucosal Colonization by Group A Streptococci

DEBRA BESSEN* AND VINCENT A. FISCHETTI

The Rockefeller University, 1230 York Avenue, New York, New York 10021

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A major virulence factor of group A streptococci is M protein, a surface-exposed fibrillar molecule of which there exist more than 80 distinct serological types. Antigenic variability resides largely in the amino-terminal region of M protein, whereas the carboxy-terminal half of the molecule is highly conserved among different M serotypes. We sought to determine whether mucosal immunization with conserved epitopes of M protein influences the course of mucosal colonization by group A streptococci in a mouse model. Synthetic peptides corresponding to sequences in the conserved region of M protein were covalently linked to the mucosal adjuvant cholera toxin B subunit. Mice were immunized intranasally with the peptide-cholera toxin B subunit conjugate or with cholera toxin B subunit alone and then challenged intranasally with live streptococci. Pharyngeal colonization by streptococci was measured for up to 15 days postchallenge. Mice immunized with synthetic peptides showed a significant reduction in colonization compared with the control group. The data demonstrate that immunity evoked by conserved portions of M protein influences the outcome of group A streptococcal infection at the nasopharyngeal mucosa in a mouse model.

Group A streptococci can cause pharyngitis in humans, a disease involving inflammation of the nasopharyngeal mucosa and underlying tissue, and ineffective antibiotic treatment can lead to rheumatic fever in up to 3% of those infected (6). A major surface component of group A streptococci is M protein, an alpha-helical coiled-coil dimer of which there exist more than 80 distinct serological types (24, 28). The amino-terminal portion of the fibrillar M protein molecule contains the antigenically variable determinants of type specificity (3, 20). Homology among M molecules of different serotypes progressively increases at sites which are closer to the carboxy terminus and more proximal to the cell wall (16, 22). A major portion of the *emm-6* gene consists of three large regions of tandem repeats, and homologous recombination between intragenic repeats generates M protein size variants at a high frequency (12, 17). M protein is considered a major virulence factor, largely on the basis of its ability to render the streptococcus resistant to nonimmune phagocytosis (5, 27).

Serum antibody directed to M protein can initiate bactericidal activity against streptococci suspended in whole blood by fixing complement, which in turn leads to opsonophagocytosis of the organisms by polymorphonuclear leukocytes (19). However, with few exceptions, opsonizing antibodies are limited to those with specificity for the antigenically variable, type-specific portion of M protein (13, 24). Type-specific immunity is considered protective, and it is rare that an individual becomes reinfected with a serotype from a previous infection. Thus, one approach for a streptococcal vaccine involves the incorporation of M antigens corresponding to several distinct type-specific determinants (2). However, development of a type-specific vaccine may be a formidable task because of the enormous array of existing M serotypes.

A general strategy for vaccine development is to raise protective antibodies against antigenic sites which are common to the many serological variants of a given microorganism. The decreased incidence of streptococcal pharyngitis in adults (6) might be explained by an age-related host factor. Alternatively, protective antibodies directed to antigens common to all group A streptococcal serotypes might arise as a consequence of multiple infections experienced during childhood. We have explored the second hypothesis and have found that of those adults examined, most have a strong antibody response to the conserved portion of M protein (D. Bessen and V. A. Fischetti, *in* L. Lasky, ed., *Technological Advances in Vaccine Development*, in press; V. A. Fischetti and M. O. Windels, J. Immunol., in press). While antibodies directed to conserved epitopes fail to neutralize the antiphagocytic property of M protein (20), they might afford protection by an alternative mechanism.

Secretory immunoglobulin A (sIgA) provides the host with a first line of defense against bacterial infection and blocks attachment of microorganisms to the mucosal epithelium (1). We have shown previously that passively acquired sIgA directed to M protein significantly inhibits streptococcal infection in experimental animals when administered mucosally (4; Bessen and Fischetti, in press). The finding that animals can be protected against streptococcal infection with sIgA, an antibody which does not opsonize, suggests that opsonophagocytosis initiated by type-specific serum IgG is not the sole mechanism by which protection can occur. This, in turn, raises the possibility that antibodies directed to non-type-specific determinants can provide initial defense against infection. Using a mouse model, we sought to determine whether conserved epitopes of M protein could elicit immunity and thereby alter the course of infection by group A streptococci. Animals were immunized intranasally (i.n.) with synthetic peptides covalently linked to the mucosal adjuvant cholera toxin B subunit (CTB) (26). We report that immunization with synthetic peptides corresponding to conserved sequences of M protein results in a significant decrease in the incidence of pharyngeal colonization by group A streptococci.

* Corresponding author.

TABLE 1. Peptides contained in the vaccine

Peptide position[a]	Sequence
216–235	SKQDIGALKQELAKKDEGNKC
248–269	LDASREAKKQVEKDLANLTAELC
275–284	EKQISDASRQC

[a] Amino acid residue numbers are based on the amino acid sequence of the mature M6 protein (15). The pepsin-susceptible site is located between residues 228 and 229.

MATERIALS AND METHODS

Peptides. On the basis of the nucleotide sequence of the *emm-6* gene (15), three peptides corresponding in amino acid sequence to the M6 protein (Table 1) were synthesized with an additional cysteine residue at the carboxy terminus by solid-phase synthesis at The Rockefeller University Sequencing Facility and purified as previously described (20). To eliminate disulfide bridges formed upon storage, before conjugation to CTB the peptides were reduced with β-2-mercaptoethanol and the reducing agent was eliminated by several cycles of lyophilization.

Conjugation of peptides to CTB. Highly purified CTB in phosphate-buffered saline, pH 7.2, was kindly provided by Institut Merieux (Lyon, France) and was largely in a pentameric form. Primary amino groups of CTB were derivatized by addition of a 15 M excess of the heterobifunctional cross-linking agent *N'*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Pierce Chemical Co., Rockford, Ill.). The mixture was continuously stirred with a magnetic stirrer until a precipitate formed (about 10 to 15 min). The reaction was stopped by addition of ethanolamine, which resulted in immediate clearance of the precipitate, and the solution was dialyzed. A small sample of the dialysate was removed, and the A_{343} was measured before and after addition of dithiothreitol to establish the extent of derivatization on the basis of release of pyridine-2-thione (7). Dialyzed CTB was mixed with a single peptide at a 1:1.5 (wt/wt) ratio at room temperature for 4 h and then overnight at 4°C. The average number of peptide molecules covalently linked per CTB monomer ranged between 1.12 and 1.20 based on A_{343} of the mixture. This degree of substitution preserved nearly 100% of the CTB binding capacity to ganglioside GM_1 compared with underivatized CTB (25); increased derivatization of CTB with SPDP led to a marked decrease in GM_1 binding capacity (data not shown).

Preparation of vaccine. Free peptide was not separated from peptide-CTB conjugates, and the entire mixture containing free peptide plus covalently linked peptide-CTB was used for immunization. The three individual peptide-CTB conjugates (containing unbound peptide) were pooled in equal weight quantities, and samples were stored at −80°C until use. Each dose of the peptide-CTB vaccine contained 20 μg of CTB plus a total of 30 μg of peptide. In one of the four vaccine trials performed, peptide(248-269) was omitted from the peptide-CTB vaccine. For control groups which received CTB only, each dose contained 20 μg of CTB.

Immunization. Mice were immunized i.n. with either the peptide-CTB mixture or CTB alone, three times over a 6-day period. Animals were rested 3 weeks and boosted i.n. with a single dose of antigen. The vaccine was delivered to the nares of unanesthetized mice (10 μl per nostril) through a Hamilton syringe (model 750) fitted with a repeating dispenser (PB600) and a blunt-end needle. Outbred Swiss CD1 mice (Charles River) were female and were 4 to 5 weeks old at the onset of immunization. Four additional mice, used for antibody studies only, were immunized subcutaneously (s.c.) with 75 μg of the purified native M6 protein (ColiM6; [11]) in complete Freund adjuvant, boosted at 4 weeks with 75 μg of ColiM6 in incomplete Freund adjuvant, and bled 10 days later.

Streptococcal challenge and follow-up. Type 6 streptococci (strain S43/192) were selected for resistance to 200 μg of streptomycin per ml. Mouse virulence of S43/192 was maintained by several intraperitoneal passages (24). A single stock of organisms was prepared from an overnight culture, concentrated 10-fold, frozen at −80°C, and used for all challenge experiments. Stocks were diluted 1:500 and grown overnight at 37°C in Todd-Hewitt broth and then diluted 1:20 in fresh growth medium. When cultures reached an optical density at 650 nm of 0.5 (18-mm-diameter tube), they were centrifuged and suspended in saline to one-sixth the volume (approximately 2.5×10^8 CFU/ml). The dosage of streptococci used for challenge was selected on the basis of its ability to reproducibly colonize greater than 25% of a nonimmune mouse population. A peptide-CTB-vaccinated group of mice was compared with a control group (CTB only) in four separate challenges with live streptococci. In each challenge experiment, the peptide-immunized and control groups contained 12 to 14 mice apiece; animals were housed four or five per cage with members of the same group. Mice were administered 10 μl of the streptococcal suspension per nostril at 10 days after boost. Throats were swabbed (Calgiswab type 4; Spectrum Laboratories, Inc.) beginning 24 h after challenge and at 24- or 48-h intervals thereafter until day 10 or 11; an additional throat culture was taken on day 14 or 15. Throat swabs were cultured on blood agar plates containing 200 μg of streptomycin per ml. Cultures displaying one or more beta-hemolytic colonies were scored as positive.

Immunological studies. Several immunized mice from each group were selected for collection of saliva and serum samples at the time of challenge of that group; these animals were eventually sacrificed and did not receive live streptococci. Whole saliva was collected after stimulation with pilocarpine (20 μg per mouse) injected s.c. and centrifuged at 15,000 × *g* for 20 min, and protease inhibitors were added to the supernatant. Saliva or serum was pooled from a total of seven mice which had been immunized with the peptide-CTB vaccine or CTB alone. In addition, saliva or serum was pooled from four mice immunized with ColiM6 (s.c.) as described above and from six mice which had received no antigen.

k-ELISA. Kinetic enzyme-linked immunoabsorbent assay (k-ELISA) (31) was performed as follows. Microdilution wells were coated with antigen at 5 μg/ml, and antibody incubations were performed as previously described (20), except that buffers contained 0.5 M NaCl. A_{405} was automatically recorded at 4-min intervals at room temperature with intermittent shaking on an ELIDA-5 reader (Physica Inc.) programmed to calculate the kinetics of the reaction in individual wells. Results are expressed in absorbance per hour. Background absorbance-per-hour measurements were determined by antibody reactivity in a well devoid of antigen and were subtracted from the reported values.

Antibody absorption assay. Heat-killed streptococci were prepared from 31 of 60 different serotypes among types 1 to 67 as described previously (21); serotypes M1, M2, M9, M11, M15, M18, M22, M25, M32, M33, M35, M36, M38, M39, M40, M43, M44, M46, M48, M51, M52, M54, M55, M56, M59, M62, M63, M65, and M66 were excluded from analyses because of nonimmune absorption of rabbit IgG

(greater than 15% absorbed). Heat-killed streptococci were adjusted to an optical density at 650 nm of 0.1 and concentrated 50-fold in 1.0% acidified bovine serum albumin in phosphate-buffered saline. Antiserum was raised in rabbits to synthetic peptides corresponding to residues 216 to 235 or 248 to 269, which had been covalently linked to ovalbumin, and affinity purified with the respective peptide as previously described (20). The concentration of affinity-purified antibody and monoclonal antibody giving half-maximal immunoreactivity to ColiM6 antigen was established by k-ELISA as described above. Heat-killed streptococci (at an optical density at 650 nm equivalent to 4.0) were mixed with antibody (at half-maximal reactivity to ColiM6) in a volume of 100 μl in V-bottom-well microdilution plates which had been preblocked with acidified bovine serum albumin. Plates were sealed, mixed 1 min on a Micro-Shaker (Dynatech Laboratories, Inc., Alexandria, Va.), and rotated end-over-end overnight at 4°C. Microdilution plates were centrifuged, and the supernatants were removed, mixed 1:1 with 0.5% Brij–20 mM phosphate (pH 7.2)–1.0 M NaCl, and transferred to flat-bottom microdilution plates which had been coated with ColiM6 antigen. Immunoreactivity was measured by k-ELISA, and all measurements were performed in triplicate. Binding of anti-M protein-specific antibodies was considered positive if more than 20% of the immunoreactivity was absorbed by the organism.

RESULTS

Antigenic epitopes of M protein. The peptides used for vaccination (Table 1) were selected on the basis of their location within regions of the M6 molecule which are known to be shared among multiple M serotypes (21, 22, 30). These peptides are located at and on the C-terminal side of the pepsin-susceptible site of the M6 molecule (12). The relative distribution of antipeptide antigenic sites on the surfaces of streptococci of other serotypes was examined in detail. Whole, killed organisms of 31 different M serotypes were tested for the ability to bind affinity-purified, antipeptide antibody raised in rabbits. Antipeptide(216-235) bound to 20% of the serotypes tested, whereas antipeptide(248-269) bound to 68%. Monoclonal antibodies 10B6 and 10F5, whose binding sites on the M6 molecule have been mapped to residues 275 to 289 (21), collectively bound to 71% of the serotypes tested. Thus, antigenic epitopes located in the central and carboxy-terminal regions of M6 protein are shared among other streptococcal types and are partially conserved. In addition, none of the antibodies which bound to multiple serotypes was capable of opsonizing type 6 streptococci in whole blood (20), and thus, the cross-reactive antibodies failed to neutralize the antiphagocytic property of M6 protein.

Pharyngeal colonization of immunized mice. Mice which had been immunized i.n. with synthetic peptide-CTB conjugates (containing free plus bound peptides) or given CTB only were challenged i.n. with live type 6 streptococci. Throat cultures were taken at 24- or 48-h intervals until day 10, with additional cultures up to day 15. Streptococcal colonization of the pharyngeal mucosa of individual mice from four separate challenges is summarized in Fig. 1 and 2. Animals which had received the peptide-CTB vaccine displayed a lower incidence of pharyngeal colonization during the 15 days following streptococcal challenge. This is evident both in the total number of animals which displayed positive throat cultures on a given day (Table 2) and in the types of colonization patterns exhibited by individual mice over the 15-day observation period (Table 3).

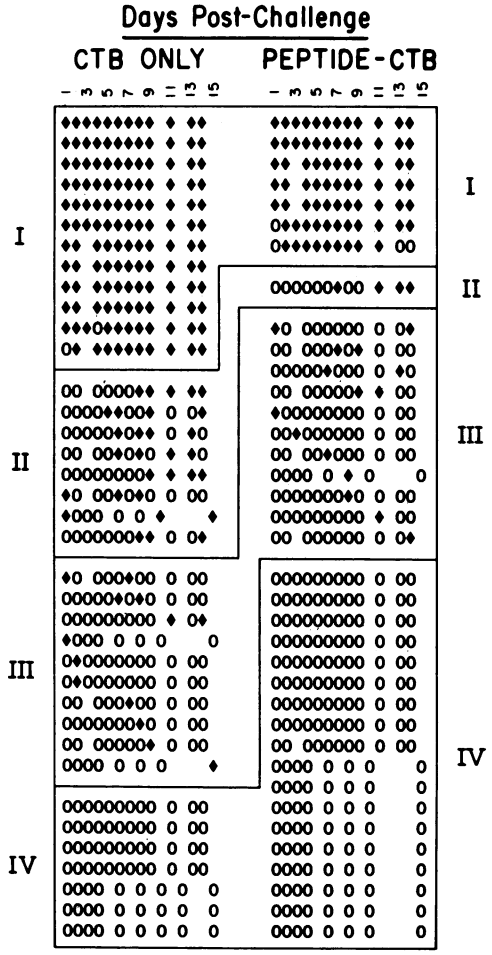

FIG. 1. Pharyngeal colonization of mice which survived i.n. challenge with streptococci. Pharyngeal cultures were taken from mice which had been immunized with CTB only or peptide-CTB for 15 days following i.n. challenge and were scored as negative (○) or positive (◆) for beta-hemolytic streptococci. The throat culture results from a total of 37 individual mice from each group are presented. Each animal was swabbed at 8 to 12 time points, depending on the experiment. Pharyngeal colonization patterns I through IV are indicated and are defined as follows: I (heavy colonization), streptococcal isolates at nearly every throat culture; II, positive culture at three to five time points; III, positive culture at one or two time points only; and IV (completely free), no isolates at any culture.

Streptococcal challenge was performed in four separate experiments. In each experiment, the peptide-CTB-immunized and CTB control groups of mice contained 12 to 14 mice apiece. The results of the four experiments are combined for the seven time points at which pharyngeal cultures were taken in all experiments (Table 2). The difference in colonization between peptide-immunized mice and the control group (CTB only) was significant for five of these seven time points (Table 2). During the course of the four challenge experiments, a total of 43 throat culture analyses were taken during the 15-day observation period. Comparison of the vaccinated and control groups at each of the 43 throat culture analyses revealed that the number of positive throat cultures plus dead among the control group exceeded that of

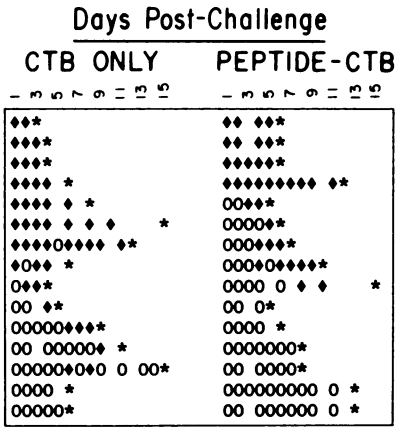

FIG. 2. Pharyngeal colonization of mice which died. Pharyngeal cultures were taken from mice which had been immunized with peptide-CTB or CTB only after i.n. challenge and were scored as negative (○) or positive (◆) for beta-hemolytic streptococci. Time of death is indicated by an asterisk. In preliminary studies, autopsies were performed on animals which had received live streptococci by the i.n. route and died; beta-hemolytic streptococci were isolated from the blood, lungs, or spleens of all animals.

the peptide-CTB immunized group 86% (37/43) of the time (data not shown). Thus, from experiment to experiment there was a consistent trend of reduced infection among peptide-immunized mice at nearly all time points.

The majority of surviving mice displayed one of four distinct patterns of pharyngeal colonization (Fig. 1 and Table 3). Of the survivors which were vaccinated with peptide-CTB, 49% remained completely free of streptococci at each throat culture (pattern IV), compared with only 19% of animals in the CTB control group. In contrast, heavy colonization (pattern I) was observed in 32% of the survivors which received CTB only, compared with only 19% of the peptide-immunized mice. In addition, 93% of the throat cultures collected from heavily colonized animals of both groups exhibited 25 or more CFU (data not shown). The majority of the remaining survivors typically had positive throat cultures at one or two time points only (pattern III), and 86% of these cultures showed fewer than 10 colonies (data not shown). Thus, the method for analyzing pharyngeal infection is highly reproducible, and most survivors either were in a stable state of heavy streptococcal colonization,

TABLE 3. Patterns of pharyngeal colonization in survivors

Pharyngeal culture pattern[a]	No. of survivors (%)	
	CTB only	Peptide-CTB
I	12 (32)	7 (19)
II	8 (22)	1 (3)
III	10 (27)	11 (30)
IV	7 (19)	18 (49)

[a] Pharyngeal culture patterns as defined in the legend to Fig. 1.

passed through a transient state of low colonization, or were completely organism free.

Human nasopharyngeal infection with group A streptococci usually remains locally confined. In contrast, streptococci administered i.n. to mice can cause death by invading the mucosal barrier and disseminating to distal sites (4, 18, 23). The rate of mortality due to systemic streptococcal infection was similar for mice which had been immunized with peptide-CTB and mice immunized with CTB only (Table 2 and Fig. 2). At 6 days after challenge the overall mortality rate was 15%, and by day 15, 29% (15/52) of the animals from each group had died. Despite similar rates of mortality, those peptide-CTB-immunized individuals which eventually died had a lower incidence of pharyngeal colonization in the days preceding death (Fig. 2). Among the subpopulation of animals which died within 15 days postchallenge, 40% of the peptide-immunized group had negative throat cultures at every time point, whereas only 13% of their control group counterparts had completely negative cultures. In addition, 47% of the control group and 27% of the peptide-immunized animals exhibited positive pharyngeal cultures at every swab point preceding death. Thus, the patterns of pharyngeal colonization among the subpopulation of animals which died reflect the patterns exhibited by animals which survived (Fig. 1 and Table 3).

Antibody response to immunization. At the time of streptococcal challenge, seven mice from each of the peptide-CTB-immunized and CTB control groups were sacrificed and the level of antigen-specific immunoreactivity was measured for pooled serum and salivary antibodies by k-ELISA. Included in this analysis were mice which had received no antigen and mice immunized s.c. with native M6 protein (ColiM6) in Freund adjuvant. Mice immunized i.n. with either peptide-CTB or CTB alone displayed a strong serum IgG response to CTB antigen compared with animals which

TABLE 2. Reduction in pharyngeal colonization induced by conserved synthetic peptides[a]

Day(s) postchallenge	No. of mice colonized and dead					
	CTB only (control)			Peptide-CTB		
	No. positive	Total dead	No. positive/no. of survivors (%)	No. positive	Total dead	No. positive/no. of survivors (%)
1	23	0	23/52 (44)	11	0	11/52 (21)
2	22	0	22/52 (42)	11	0	11/52 (21)
4	17	4	17/48 (35)	14	0	14/52 (27)
6	22	9	22/43 (51)	12	7	12/45 (27)
8	23	10	23/42 (55)	12	10	12/42 (29)
10 and 11	19	12	19/40 (48)	12	11	12/41 (29)
14 and 15	19	15	19/37 (51)	9	15	9/37 (24)

[a] Animals were immunized with peptide-CTB conjugates or CTB only and challenged with live streptococci in four separate experiments (52 mice per group). Throat cultures were taken for up to 15 days postchallenge and were scored for the presence of beta-hemolytic streptococci. The number of mice which died during the course of the experiment is indicated, and the number of survivors was calculated by subtracting the dead from the prechallenge population. The proportions of survivors which were positive for pharyngeal colonization in the peptide-CTB-immunized and control groups were compared. Statistical difference was significant ($P < 0.025$) on days 1, 2, 6, 8, and 14 and 15, according to chi-square analysis.

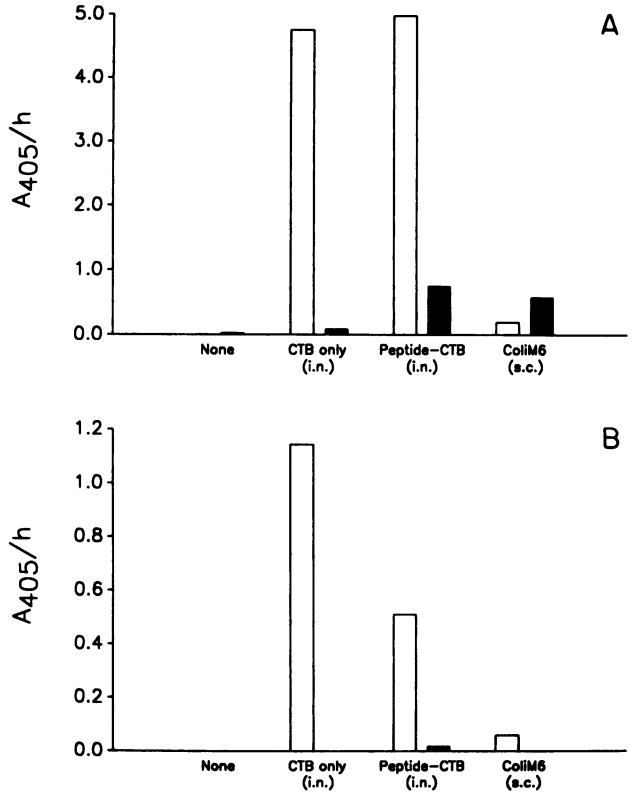

FIG. 3. k-ELISA analysis of serum IgG (A) and salivary IgA (B) reactivity. Antibody from mice which had received no antigen, CTB only (i.n.), peptide-CTB (i.n.), or ColiM6 (s.c) was tested against CTB (□) and ColiM6 (■) antigen by k-ELISA. Serum samples were tested at a 1:20 dilution. Saliva was tested at 90% of its original concentration. Immunoreactivity is expressed in A_{405} per hour. Anti-CTB reactivity was not determined for animals which received no antigen. The secondary antibodies anti-IgG and anti-IgA, used for panels A and B, respectively, were used at immunoreactivities which were not equivalent for equal quantities of their respective antigens (specific activity of anti-IgG exceeded that of anti-IgA); therefore, comparisons between the magnitudes of the IgA and IgG responses are not relevant.

did not receive CTB (Fig. 3A). Mice immunized i.n. with synthetic peptides corresponding to the M6 protein displayed an elevated level of serum IgG directed to the native M6 molecule (Fig. 3A). This degree of immunoreactivity was comparable to that observed with animals injected with M6 protein in Freund adjuvant. However, in mice immunized with peptide-CTB, the magnitude of the serum IgG response to M6 protein was sevenfold lower than that of the serum IgG response to CTB. The elevated levels of antigen-specific serum IgG after i.n. immunization observed in this study appear to parallel findings by others that oral immunization with whole cholera toxin does not lead to oral tolerance and, in addition, that such immunization circumvents systemic unresponsiveness to coadministered antigen (10).

Mice immunized with either peptide-CTB or CTB alone displayed a strong salivary IgA response to CTB antigen compared with animals which did not receive CTB (Fig. 3B). This finding is consistent with reports by others that whole cholera toxin and CTB are among the most potent, nonviable mucosal antigens known (10, 26). In contrast, the salivary IgA response to M6 protein was only slightly elevated in mice which had been immunized i.n. with peptide-CTB and was near the limit of detection of this assay. However, the elevation in anti-CTB salivary IgA titers is indicative that a mucosal immune response to i.n. immunization did in fact occur. On the basis of results of capture ELISA, we estimate that the salivary IgA responses to CTB antigen in control and peptide-CTB-immunized mice are 20 and 10%, respectively, of the total salivary IgA. The IgA immunoreactivity to ColiM6 antigen in mice which received peptide-CTB conjugate represents approximately 0.5% of the total salivary IgA.

DISCUSSION

The goal of this study was to determine whether mucosal immunization with surface-exposed, non-type-specific epitopes of the M protein molecule could modulate the course of group A streptococcal infection. The synthetic peptides chosen for immunization correspond to antigenic epitopes shared by many distinct M protein types, and therefore the immunogens do not represent type-specific determinants. Our findings indicate that i.n. immunization with synthetic peptides corresponding to cross-reactive epitopes of M protein leads to significant protection against pharyngeal colonization by streptococci in a mouse model. The lower incidence of pharyngeal colonization among peptide-immunized mice was evident both in the number of animals displaying positive throat cultures on a given day and in the type of colonization pattern exhibited by individual mice. The decreased incidence of initial colonization (days 1 and 2 postchallenge) in peptide-immunized mice is in support of the hypothesis that protection is occurring at the mucosal level, before infection is firmly established. Thus, i.n. immunization with conserved synthetic peptides leads to a reduction in group A streptococcal colonization, and the findings strongly suggest that protective immunity occurs at the mucosal level.

It is widely accepted that only type-specific serum antibody protects mice against systemic infection and death after intraperitoneal challenge with group A streptococci (13, 24). Intranasal administration of live organisms to mice can also cause disseminated streptococcal disease. We and others (18, 23) have isolated streptococci from the lungs and spleens of dead animals which had been given live organisms by the i.n. route. In this study, penetration of the mucosal barrier and subsequent systemic infection and death were not altered in mice immunized with non-type-specific M protein antigens. The inability of non-type-specific immunity to protect against disseminated infection in the mouse is in agreement with other findings (13, 24). The absence of pharyngeal isolates among many mice which succumbed to systemic infection suggests that streptococci can penetrate the mucosal barrier at sites other than the pharynx. In addition, the incidence of pharyngeal colonization in the days preceding death was lower for the peptide-immunized mice compared with the control group and paralleled the incidence of colonization and pharyngeal culture patterns of their surviving counterparts. Furthermore, the equal number of deaths among vaccinated and unvaccinated mice confirms that an equivalent number of streptococci was received by both groups, despite their striking differences in colonization. In conclusion, the data strongly suggest that the ability of streptococci to penetrate the mucosal lining is independent of the degree of colonization at the pharyngeal mucosa in this mouse model.

The inefficiency of non-type-specific serum IgG in promoting opsonophagocytosis of group A streptococci (20) has

provided the impetus to search for alternative mechanisms of protection. Previous studies have shown that when anti-M6 protein sIgA is passively administered at an intranasal site, mortality due to type 6 streptococcal challenge i.n. is significantly reduced (4). In contrast, anti-M6 serum antibody, which is both opsonic and 30-fold more immunoreactive with M6 protein than the sIgA, is not protective at this mucosal site (4). In earlier vaccine trials, humans immunized with M protein at an i.n. site displayed low rates of nasopharyngeal colonization after challenge with streptococci of the homologous M type compared with recipients of a parenterally administered vaccine (9, 14, 29). Therefore, we sought to stimulate a secretory antibody response in mice by immunizing at a mucosal site. Despite a significant decrease in pharyngeal colonization in animals which received the synthetic peptide vaccine corresponding to conserved sites on M protein, salivary IgA titers to M protein remained low. The level of anti-M protein sIgA necessary for protection against streptococcal infection in an actively immunized host is not known, and it may be that very small quantities of sIgA can protect against colonization. Perhaps the rise in serum IgG titer to M6 protein in peptide-CTB-immunized mice reflects a similar response which occurred at the mucosa, and k-ELISA analysis is not sufficiently sensitive to detect this quantity, even though k-ELISA is more sensitive than endpoint ELISA (31). The increase in CTB-specific salivary IgA indicates that a mucosal immune response to immunization did in fact occur. Nevertheless, we cannot state with certainty that anti-M protein sIgA was involved in protection against mucosal streptococcal infection in this study, nor can we exclude a possible role for non-type-specific IgG.

The incidence of streptococcal pharyngitis in adults is substantially lower than that in children. Most adults tested have a strong serum IgG response to conserved regions of M protein, and this probably arises as a consequence of multiple streptococcal exposures earlier in life (Bessen and Fischetti, in press; Fischetti and Windels, in press). Each peptide component of the vaccine tested in this study was found to be conserved among many distinct serological M types. When rabbits are given a single injection of the native M6 molecule or killed type 6 streptococci in Freund adjuvant, the serum IgG response to M6 protein is directed largely to peptides contained within the B repeat region of M6 protein (Fischetti and Windels, in press), a series of tandemly arranged, 25-amino-acid segments which are repeated in whole or part (15). The sites of the peptides used for mouse immunization in this report lie immediately adjacent to the carboxy terminus of the immunodominant B repeat region and appear to be relatively immunorecessive when presented in the form of the native M6 molecule. The immunological targeting of epitopes which are immunorecessive when presented within the native molecule may be an effective strategy for vaccine development, and synthetic peptides may be particularly well suited to this approach. To develop a safe and effective peptide vaccine against human nasopharyngeal infection by group A streptococci, we intend to better identify those peptides which provide the broadest protection without stimulating adverse effects, such as an autoimmune response to human heart tissue (8).

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ADDENDUM IN PROOF

The recent article by Miller et al. (L. Miller, V. Burdett, T. P. Poirier, L. D. Gray, E. H. Beachey, and M. A. Kehoe, Infect. Immun. **56**:2198–2204, 1988) suggests that antibodies to conserved M protein epitopes located on the C-terminal side of the pepsin cleavage site do not bind to intact streptococci, and therefore broadly conserved epitopes are inaccessible to antibody. In several studies employing a variety of assays with both monoclonal and anti-peptide antibody probes, we found that broadly conserved epitopes located on the C-terminal side of the pepsin site of M protein are readily accessible to antibody when presented on the surface of intact streptococci (20–22, 26a; also this study and D. Bessen et al., manuscript in preparation). While conditions may exist wherein conserved M protein epitopes are partially or completely masked, our evidence clearly leads us to conclude that epitopes located adjacent to the pepsin-susceptible site and in the C repeat block region of M6 protein (amino acid residues 229 to 298) are, for the most part, immunoaccessible on intact streptococci and broadly cross-reactive.

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