INFECTION AND IMMUNITY, June 1991, p. 2158–2165
0019-9567/91/062158-08$02.00/0

Vol. 59, No. 6

Expression and Immunogenicity of a Streptococcal M Protein Epitope Inserted in *Salmonella* Flagellin

SALETE M. C. NEWTON,[1] MALAK KOTB,[2,3,4] THOMAS P. POIRIER,[4] BRUCE A. D. STOCKER,[1]* AND EDWIN H. BEACHEY[3,4,5]†

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305,[1] and Departments of Surgery,[2] Microbiology and Immunology,[3] and Medicine,[5] The University of Tennessee at Memphis, and the Veterans Administration Medical Center,[4] Memphis, Tennessee 38104

Received 17 December 1990/Accepted 26 March 1991

A synthetic 48-bp oligonucleotide specifying the N-terminal 15 amino acids of M protein of *Streptococcus pyogenes* type 5 (plus a CTA codon, to terminate translation of genes with the insert in reverse orientation) was inserted by blunt-end ligation at the site of the 48-bp *Eco*RV deletion in the *Salmonella* flagellin gene in plasmid pLS408 (S. M. C. Newton, C. O. Jacob, and B. A. D. Stocker, Science 244:70–72, 1989). The resulting plasmid was transferred from *Escherichia coli* via a restriction-negative *Salmonella typhimurium* strain into an aromatic-compound-dependent, flagellin-negative live-vaccine strain of *Salmonella dublin* to produce strain SL7127, which was motile. Expression of the inserted epitope in flagellin and its exposure at the flagellar filament surface were shown by immunoblotting and by the reaction of flagellate bacteria (immobilization, immunogold labeling) with antibody raised by injection of the corresponding synthetic peptide, S-M5(1-15). Rabbits immunized by injection of the live-vaccine strain with flagella composed of the chimeric flagellin or by injection of concentrated flagella from such bacteria developed antibodies reactive in an enzyme-linked immunosorbent assay with peptide S-M5(1-15) and with the large peptic-digest peptide pepM5. These antibodies were opsonic for type 5 streptococci. Mice that were given parenteral live SL7127 (six doses, each 1×10^6 to 2×10^6, over 8 weeks) developed titers of ca. 12,800 for the M5-specific peptides and opsonizing activity for type 5 streptococci but not for type 24 streptococci. Sera from mice similarly immunized with a control live vaccine strain without an insert in the flagellin gene did not react with the M5-specific antigens. All of the five mice given the control strain, without an insert, died after challenge with type 5 streptococci or type 24 streptococci; by contrast, four of the five mice given strain SL7127, with an insert, survived the M5 challenge, but none of the five challenged with the type 24 strain survived. Therefore, our study shows that an M protein epitope can be expressed in the context of an unrelated protein and maintain its immunogenicity. Furthermore, we demonstrate that mice can be protected against a *Streptococcus pyogenes* type 5 challenge by immunization with a *Salmonella* live vaccine with flagella made of flagellin with an insert carrying a protective epitope of M5 protein but without the cross-reactive epitopes of the complete protein.

M protein, which makes up the α-helical fibrils that extend outward from the surface of group A *Streptococcus pyogenes*, is a major virulence determinant of streptococci; it has antiopsonic properties that enable the organism to escape attack by phagocytic host cells (21, 28). Antibodies to M protein protect the host by opsonizing the bacteria and killing them by phagocytosis (16, 31, 32). A significant proportion of persons infected with certain strains of group A streptococci develop autoimmune diseases such as rheumatic fever and rheumatic heart disease. The finding of group A streptococcal antigens that are cross-reactive with human heart tissue (12, 15, 18, 30, 35) has hampered the development of vaccines, since immunization with such antigens might elicit autoimmune reactions. Initial studies to characterize these cross-reactive antigens from *S. pyogenes* indicated that most were associated with cell wall or cell membrane components (13, 14, 30, 34). However, recent studies showed that purified M protein evokes antibodies that cross-react with sarcolemmal membranes of human heart muscle (7–9). Experiments with synthetic peptides corresponding to various regions of M protein revealed that the amino-terminal regions of type 5 and 6 streptococcal M proteins contain protective but not cross-reactive epitopes (1–5). Therefore, we directed our efforts toward devising synthetic or recombinant vaccine preparations that were free of autoimmune determinants. The advantage of recombinant vaccines is that various chimeric constructs of protective epitopes from several M proteins can readily be made by genetic manipulations and that lower doses of M protein might be sufficient to produce a protective immune response. To this end we sought several relatively innocuous delivery vehicles that are also potent immunogens in which the protective epitopes of the M protein can be inserted.

In a previous study we were able to express M protein in a live-vaccine strain of *Salmonella typhimurium*, SL3261, and test its protective effect by giving it to BALB/c mice via the oral route (22). Animals given strain SL3261 expressing the complete M protein gene made opsonic antibodies and were protected against challenge with the homologous *S. pyogenes* type 5 strain but not against a type 24 strain. Furthermore, SL3261-delivered M5 protein failed to elicit antibodies that cross-reacted with heart tissue, presumably as a consequence of the different mode and/or site of delivery and the relatively low M protein dosage.

Flagellar filaments have recently been used as carriers of unrelated epitopes by insertion of epitope-specifying oligonucleotides at a restriction site in a region of the gene coding for flagellar-antigen determinants. Some such constructs

* Corresponding author.
† Deceased, 27 October 1989.

elicited humoral responses to the foreign epitope in several species of experimental animals (20, 33); the response was not dependent on normal flagellar structure or function (33). In this report, we describe the humoral and cellular immune responses of mice and rabbits to a protective, non-cross-reactive epitope from *S. pyogenes* type 5 M protein inserted in flagellin in such a way that it was exposed at the surface of the filament part of functional flagella. *Salmonella* live-vaccine strains expressing flagellar filament protein harboring selected epitopes from M protein might be able to confer protection against streptococcal infection without the risk of rheumatic diseases.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* CL447 is a K-12 C600, restriction-negative *fliC* (formerly *hag*) strain that is unable to make flagella (20). *S. typhimurium* LB5000 is a restriction-negative, modification-proficient strain (6, 23). *S. pyogenes* type 5 strain Smith has been described previously (2). Plasmid pLS408, a pUC19 derivative, is a small nonconjugative plasmid that includes a β-lactamase gene, so that it confers ampicillin resistance. An *Eco*RI-*Eco*RI fragment from the chromosome of *Salmonella muenchen* that includes gene *fliC*(d), specifying flagellin corresponding to flagellar antigen *d*, inserted at the *Eco*RI site of pUC19 produced plasmid pLS405; the in vitro deletion of a 48-bp *Eco*RV-*Eco*RV fragment of the flagellin gene (from the hypervariable segment, determining flagellar antigenic character) resulted in plasmid pLS408, with a single *Eco*RV site, convenient for oligonucleotide insertion. Plasmid pLS408 confers motility when introduced into *E. coli* or *Salmonella* strains that are nonmotile because they lack a functional flagellin gene (20). Strain SL5928 is an *aroA* live-vaccine strain of *Salmonella dublin* that is nonmotile because its only flagellin gene, *fliC*(g,p), has been replaced, via transduction, by *fliC*(i)::Tn*10*, an allele inactivated by transposon insertion. (Strain SL5928, used in this and previous investigations as a host for plasmid-borne flagellin genes with epitope-specifying inserts [20] was by error there stated to have been derived from strain SL1438, a motile *aroA* live-vaccine strain of *S. dublin* tested as live vaccine in calves [27]. In fact, the motile parent of SL5928 is strain SL5631, another *S. dublin* strain with a different deletion, *aroA148*, making it nonvirulent and without the *hisG46* mutation present in strain SL1438; strain SL5631 was constructed [25, 27a] for trial as live vaccine in calves.) Strain SL5930 is the flagellin-negative *aroA* live-vaccine strain SL5928 carrying plasmid pLS408, which has the 48-bp deletion in the flagellin gene but no insert; this strain was used as a control to detect antibody specific for flagellar antigen *d* or for testing the ability of *aroA* live vaccines to elicit nonspecific protection.

Purification of M protein, synthetic peptides, and antiserum production. M protein was purified from intact *S. pyogenes* type 5 strain Smith by limited pepsin digestion as described previously (3). Pepsin cleaves between the B4 and C repeats and releases the amino-terminal half of the M protein. The resulting polypeptide fragment (pepM) was further purified by ion-exchange chromatography, and its homogeneity was verified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (3). Peptide S-M5(1-15)c comprises 15 amino acids from the amino-terminal end of type 5 M mature protein (9) with an added cysteine residue to facilitate conjugation to carrier proteins. Antisera to either pepM5 or to the synthetic peptide S-M5(1-15)c conjugated to keyhole limpet hemocyanin were generated in New Zealand White rabbits as previously described (9).

Cloning strategies. Plasmid pLS408 was linearized with *Eco*RV and treated with calf intestinal phosphatase (Boehringer Mannheim) according to the recommendations of the manufacturer. Complementary 48-base oligonucleotides specifying the 15 N-terminal amino acids of type 5 M protein followed by CTA were synthesized. A *Kpn*I site facilitated recognition of recombinant plasmids, and the final CTA (Leu) codon would be read as a stop codon, preventing flagellin synthesis, in clones with the insert in the reverse orientation. The two strands were annealed at 65°C and blunt end ligated to pLS408 treated as described above with T4 DNA ligase (BioLabs). Ligations proceeded overnight at room temperature. Flagellin-negative *E. coli* CL447 was transformed by a standard procedure (17) with the ligation mixture. Transformants were selected on plates of L-ampicillin agar and screened for motility (ability to spread in semisolid medium). Plasmid preparations were made from transformants by a standard procedure (19). Recombinant plasmids were screened by *Kpn*I digestion and sequenced by double-stranded sequencing (24) with Sequenase (U.S. Biochemical) and a 15-mer primer corresponding to a sequence 30 bp downstream from the *Eco*RV insertion site in the flagellin gene.

Immunoblot analysis. *Salmonella* live-vaccine strains harboring recombinant plasmids were inoculated into semisolid medium; after overnight incubation at 37°C, loopfulls from the advancing edge of growth were inoculated into rich medium and incubated with gentle agitation for ca. 3 h, and aliquots were removed and used to make protein extracts as described by Silhavy et al. (26). Protein extracts were applied to 10% polyacrylamide–SDS gels and submitted to electrophoresis at 60 mA for 3 h. The proteins were transferred to nitrocellulose filters for 2 h in methanol buffer (29). Filters were incubated overnight at 25°C in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). Antibodies to flagellar antigen *d* or to peptide SM5(1-15) were diluted appropriately in PBS–1% BSA, added to the filters, and incubated at 25°C for 1.5 h with gentle shaking. Incubation was followed by three washes in PBS containing 0.05% Tween 20. Peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) was diluted in PBS–1% BSA and added to the filters, which were kept for 1 h at 25°C. Three more washes were made as before, and the color was developed by using 4-chloro-α-naphthol as a substrate. The reaction proceeded until bands were visualized (3 to 5 min) and was stopped by washing the filters in distilled water.

Immunoelectron microscopy. *Salmonella* live-vaccine strains harboring recombinant plasmids were grown in rich medium and fixed by addition of Formalin at 0.5% (vol/vol). Aliquots were applied to grids and left for 5 min at room temperature. Excess liquid was drained with tissue paper, and antibodies against the inserted M protein peptide, S-M5 (1-15)c, were added to the grid (1:10 dilution) in PBS–1% BSA. After 30 min of incubation, two washes with PBS–1% BSA were made, and gold-conjugated goat anti-rabbit immunoglobulin G (Janssen) was added (1:10 dilution) in PBS–1% BSA. The grids were washed three more times and then negatively stained for 1 min in 0.5% phosphotungstic acid (pH 4.0). Bacterial cells were visualized with a JEOL 1200 EX electron microscope.

ELISAs. Enzyme-linked immunosorbent assays (ELISAs) for anti-peptide M5 or anti-S-M5(1-15)c antibody were performed by using standard procedures (10). Antigens were dissolved in sodium bicarbonate buffer (pH 9.6), added to

M5 gene 5'/-GCC-GTG-ACT-AGG--GGT-ACA--ATA-AAT-GAC-CCG-CAA-AGA-GCA-AAA-GAA-/-3'

M5 protein Ala Val Thr Arg Gly Thr Ile Asn Asp Pro Gln Arg Ala Lys Glu

Insert 5' -GCT-GTT-ACC-CGT- GGT-ACC- ATT-AAT-GAT-CCG-CAG-CGT- GCT -AAA-GAA- CTA-3'

FIG. 1. Amino acid sequence of the 15 N-terminal amino acids of processed type 5M protein and base sequence of the corresponding part of the cloned *S. pyogenes* gene, for comparison with the base sequence of the synthetic oligonucleotide inserted in the flagellin gene. The *Kpn*I site (GGTACC) was incorporated for screening of recombinants. The CTA codon specifying leucine was included so that insertion in the reverse orientation would produce nonmotile clones because of the presence of a stop codon.

microtiter wells at 1 μg per well, and incubated overnight at 4°C or for 3 h at 37°C. The wells were washed nine times with 0.15 M NaCl containing 0.05% Tween 20 (saline-Tween). Antisera were diluted in PBS and added to the appropriate wells and incubated for 1 h at 37°C. After nine washes in saline-Tween, appropriate peroxidase-conjugated second antibodies were added, and the wells were incubated for 1 h at 37°C. This was followed by nine additional washes with saline-Tween. The color reaction was developed with 5-aminosalysilate as the chromophore. The ELISA titer is defined as highest dilution of test sera at which the ratio of A_{430} of the test serum to the A_{430} of the preimmune serum was >2.

Type-specific opsonic antibody assay. In vitro assays for type-specific opsonic antibodies were performed as described previously (2, 3). Opsonizing activity is defined as the percentage of neutrophils with associated streptococci after a 45-min rotation in whole heparinized blood containing preimmune or immune serum.

Isolation of flagella. Bacteria were grown with gentle agitation overnight in LB containing 25 μg of ampicillin per ml and 10 μg of *para*-aminobenzate per ml. After centrifugation the pellet was suspended in 1/10 of the original volume in saline solution. The suspension was blended in three cycles of 45 s each at maximum speed. Bacteria were centrifuged, and the clear supernatant was submitted to ultracentrifugation at 45,000 × *g* for 1 h. The pellet was suspended in 400 μl of deionized H_2O and used in Western immunoblotting experiments to confirm presence of flagellar filament protein.

Immobilization test. Serum to be tested for M5-specific immobilizing activity was incorporated in a semisolid medium at a final 1:50 dilution, and then the *Salmonella* live-vaccine strain SL7127, harboring the chimeric flagellin, was inoculated into this semisolid medium. Migration was compared with that of the same strain inoculated in plain semisolid medium. Antibodies to the peptide S-M5(1-15)c and sera from animals immunized with live-vaccine strain SL7127 absorbed with a Formalin-fixed suspension of strain SL5930 (which expresses the flagellin gene with the in vitro deletion but without any insert) were tested for the ability to immobilize strain SL7127.

Animal immunization studies. Rabbits were immunized subcutaneously with 10^8 heat-killed bacteria or with purified flagella from strain SL7127 (three doses at 4-week intervals and two doses at 2-week intervals for a total of five doses). Sera were collected from the ear vein and tested by ELISA. Opsonic antibodies were detected in sera of rabbits 12 weeks after initial injection by in vitro opsonization assays as described elsewhere (3).

In mouse protection studies, two groups of 15 mice were injected intraperitoneally over 8 weeks. After the initial immunization, the mice were boosted on weeks 1, 2, 3, 5, and 6 with 1×10^6 to 2×10^6 live vaccine, with or without the M5 epitope insert. Pooled preimmune sera and immune sera obtained before each booster inoculation were examined by ELISA for the presence of antibodies to the relevant antigens. The sera were also tested for the presence of opsonic antibodies to type 5 *S. pyogenes* (3). Fifteen days after the last injection, groups of five mice from each of the immunized groups or groups of three mice from a control unimmunized group were challenged with 1.6×10^6 type 5 streptococci, 2.2×10^5 type 24 streptococci, or 6.3×10^3 *S. dublin* SL5608 (wild type, virulent). Survival was recorded over a 7-day period.

RESULTS

Insertion of epitope-specifying oligonucleotides in *Salmonella* flagellin gene. The sequence of the oligonucleotides used is shown in Fig. 1. Ligation of the annealed 48-mer oligonucleotides to *Eco*RV-digested pLS408 and transformation into *E. coli* CL447 gave 58 ampicillin-resistant clones, 25 of which were motile. The oligonucleotides were designed to specify a stop codon when inserted in the reverse orientation; this would account for the approximately 50% nonmotile clones. The plasmids of several clones, confirmed as recombinant plasmid by the presence in the plasmid of an extra *Kpn*I site and the absence of any *Eco*RV site, were sequenced. All of four plasmids sequenced from motile clones had the M5 oligonucleotide insert in the right orientation and reading frame; one such plasmid was designated as pLS439. Sequencing of one nonmotile clone showed the presence of the M5 epitope insert in-frame but in the reverse orientation, as expected; this plasmid was designated as pLS442.

Expression of M protein epitope in flagellin in *E. coli* CL447. Protein extracts made from *E. coli* CL447 harboring recombinant plasmids were separated on polyacrylamide gel, transferred to nitrocellulose filters, and probed with antibody against flagellar antigen *d* or peptide S-M5(1-15)c. Motile clones (harboring the insert in the right orientation) produced a very faint band reacting with anti-*d* antibody. No band reacting with antipeptide serum could be detected. No bands were detected in protein extracts from the nonmotile clone (insert in reverse orientation) with either antibody (data not shown).

Transfer of recombinant plasmids to a *Salmonella* live-vaccine strain. *S. typhimurium* LB5000 (R^-/M^+) was transformed to ampicillin resistance with plasmid pLS439 or pLS442. Strain LB5000 is not a suitable host for assessing flagellar function because it has a mutated *flaA* gene, which prevents production of flagella. Recombinant plasmids, now modified for acceptance in *Salmonella* strains, were transferred to appropriate strains by transduction. Phage P22HT104/1 *int* was grown on LB5000 transformants, and the lysates were used to transduce ampicillin resistance to *S. dublin* SL5928, an *aroA* (nonvirulent) live-vaccine strain that is nonmotile because it lacks a functional flagellin gene (see

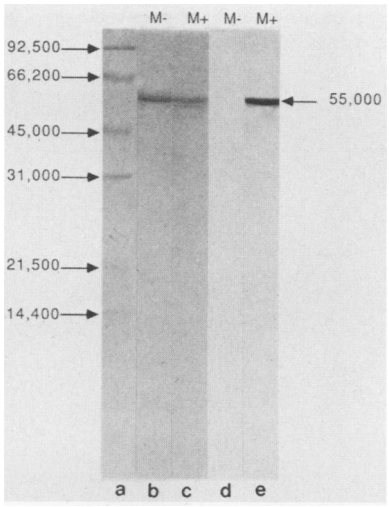

FIG. 2. Protein immunoblot analysis of *S. typhimurium* SL5928 harboring various flagellin plasmids. Bacterial protein extracts were separated by electrophoresis on 10% SDS–polyacrylamide gel and blotted on to nitrocellulose paper. Lanes: a, molecular weight standards; b and d, extract from SL5930 (without the M protein insert); c and e, extract from SL7127 (with the M protein insert); b and c, reacted with anti-*d* antibody; d and e, reacted with anti-S-M5(1-15) polyclonal antibody.

Materials and Methods). Transductants were purified and screened for motility. All transductants given plasmid pLS439 (with the M protein-specifying oligonucleotide insert) were motile. One such clone was designated as SL7127. Transductants given pLS442 (insert in reverse orientation) were nonmotile; one clone was designated as SL7130.

Expression of M protein epitopes by a *Salmonella* live-vaccine strain. Protein extracts were made from strains SL7127 (with insert), SL7130 (reverse orientation), and SL5930 (without insert). Western blot analysis with anti-*d* serum showed a band of the expected size for flagellin *d* in extracts from strains SL5930 and SL7127, but no band could be seen in the extract from strain SL7130 (reverse orientation). The antipeptide serum gave a strong reaction with a protein from strain SL7127 at the position of the material reacting with anti-*d* serum (Fig. 2). The same result was obtained using preparations of isolated flagella as protein source (data not shown).

Immunogold labeling of chimeric flagellar filament and immobilization by anti-peptide serum. *S. dublin* live-vaccine strains SL7127 and SL5930 were gold-labeled with rabbit anti-S-M5(1-15)c antiserum as the first antibody and gold-labeled goat anti-rabbit immunoglobulin G for a second antibody. The cells were examined with an electron microscope; there is strong binding only with strain SL7127, as expected, and the binding was restricted to the flagellar filament (Fig. 3).

Expression of the M5 epitope at the surface of the flagellar filament was also tested by serum immobilization. The rabbit anti-S-M5(1-15) antiserum prevented the spread of strain SL7127 in semisolid medium and arrested the translational motility of cells from a broth culture of this strain but had no such effect on strain SL5930, which lacks the M5 epitope insert in the flagellin.

Immunogenicity of M5 chimeric flagellin and flagella. Three rabbits immunized by subcutaneous injection of 10^8 heat-killed strain SL7127 cells developed antibodies reactive with peptide S-M5(1-15)c, as also did three rabbits injected with concentrated flagella from strain SL7127; the peak ELISA titers during the 3-month immunization varied from 800 to 12,800 (Table 1). The rabbits immunized with purified flagella from strain SL7127 developed a strong humoral response to peptide M5, comparable to that of rabbits immunized with S-M5(1-15)c. The sera from the SL7127-immunized rabbits also had antibodies that reacted with S-M5(1-15)c, although overall at somewhat lower titers than those of S-M5(1-15)c-immunized rabbits (Table 1). Further, two of the three rabbits vaccinated with heat-killed SL7127 and all of the three rabbits vaccinated with flagella from SL7127 developed type-specific antibody, as measured in the opsonization test (Table 1).

The serum samples from the two SL7127-immunized rabbits were extensively adsorbed with a flagellate suspension of strain SL5930, to remove antibody with affinity for the flagellar antigen. Complete adsorption was determined by the absence of immobilizing activity toward the adsorbing strain. All of the adsorbed sera inhibited motility of strain SL7127, as shown both by the effect on the rate of spread in serum-supplemented semisolid medium and by low-power dark-field microscopy, thus demonstrating the presence of an antibody with affinity for the peptide epitope.

Protective immune response to *Salmonella* live vaccine making M5 chimeric flagellin. Three groups of five mice were vaccinated with either strain SL7127 or SL5930. After the initial immunization, the mice were boosted at weeks 1, 2, 3, 5, and 6. Each dose, given intraperitoneally, comprised 1×10^6 to 2×10^6 live bacteria (no adverse effect of live-vaccine administration was noted on any occasion). Pooled sera from each group of five mice, bled before the first and each subsequent (booster) inoculation, were titrated by ELISA with synthetic peptide S-M5(1-15)c, pepM5, or bacteria of strain SL5930 (no insert in flagellin) as the coating antigen. Each serum pool was also tested for opsonization of *S. pyogenes* type 5 (strain Smith). The ELISA titers of the three pools of serum from mice given the same vaccine were either identical for each bleeding or differed by no more than one doubling dilution. The data in Table 2 show the ELISA titers and opsonic activity for each bleeding. Eight weeks (and four booster doses) after the first vaccine administration, the three pools of sera from mice given strain SL7127 (with the chimeric flagellin) had ELISA titers against each of the two M5 peptides and for whole bacteria of strain SL5930 in the range 6,400 to 12,800; each pool of sera had opsonic antibodies that caused the attachment of type 5 streptococci to human neutrophils, with 90 to 92% of neutrophils having one or more associated streptococcus after a 45-min rotation. The preimmune serum pools from the same mice had no opsonizing activity (Table 2). All three pools of sera from mice given strain SL5930, without an insert in the flagellin gene, likewise had titers of 12,800 for bacteria of strain SL5930 but even after four booster doses had insignificant ELISA titers, 100 or less, for each of the two M5 peptides and no opsonizing activity.

The high ELISA titers for the M5 peptides and the opsonizing activity of the 8-week sera indicated probable protection (Table 3). Accordingly, 2 weeks after the 8-week bleeding (and fifth booster dose) all of the vaccinated mice were challenged; for each of the two vaccine sets one group of five mice were challenged intraperitoneally with 1.6×10^6 type 5 streptococci, another group of five mice were challenged with 2.2×10^5 of type 24 streptococci, and the third group of five mice were challenged with 6.3×10^3 SL5608, the *aro*$^+$ (wild type, virulent) ancestor of the *S. dublin* strain

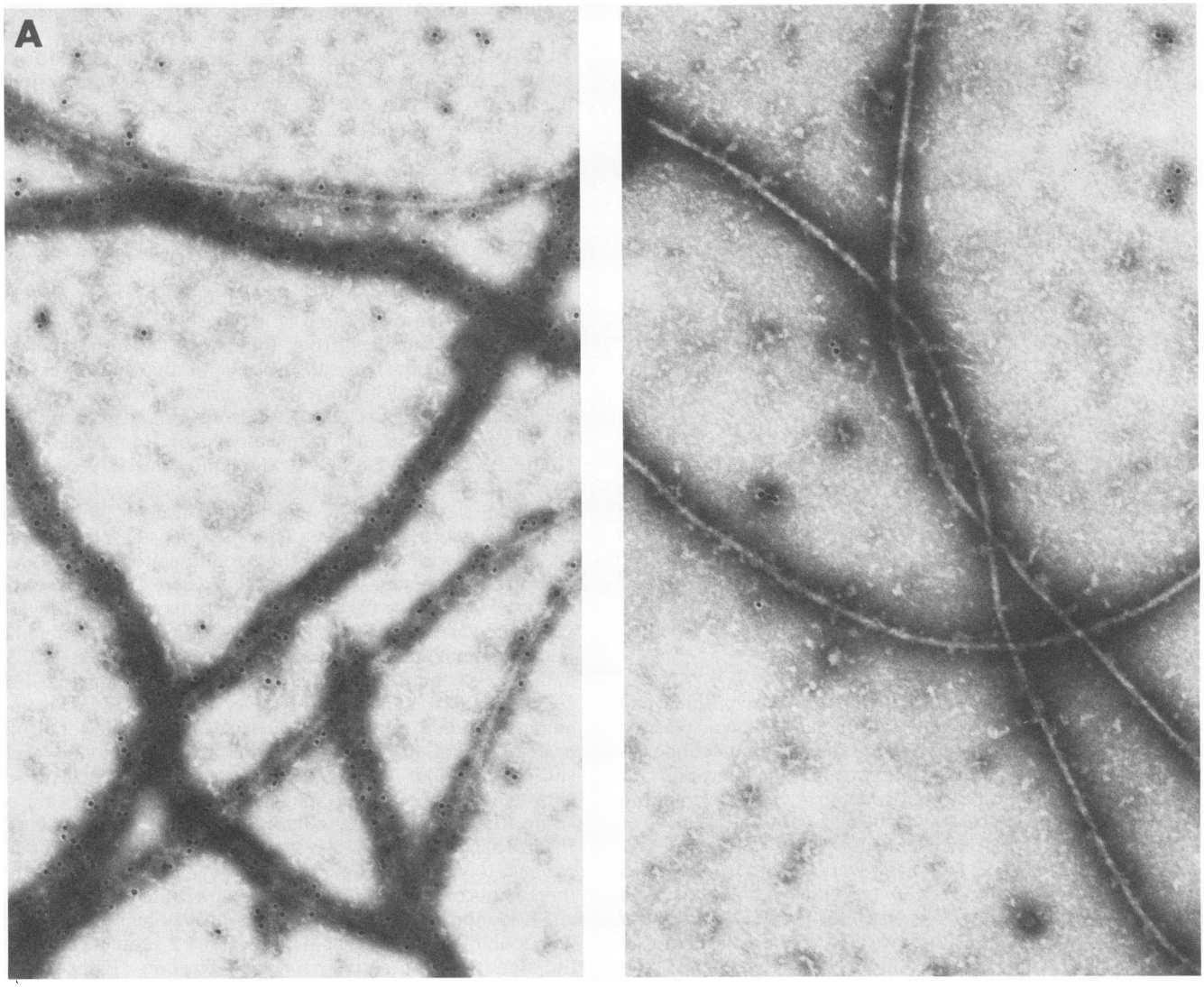

FIG. 3. Immunogold labeling of the M5 epitope at the surface of the flagella. Strain SL7127 (with the M5 insert) (A) and strain SL5930 (without the insert) (B) were labeled by treatment with anti-S-M5(1-15) and gold-conjugated goat anti-rabbit antibody as described in Materials and Methods.

used as a live vaccine. Another group of five nonvaccinated mice were given the same virulent *S. dublin* challenge. The five nonvaccinated control mice died after challenge with type 5 streptococci. As expected, none of the mice vaccinated with either strain died from the *S. dublin* challenge, which caused fatal infections in all five nonvaccinated control mice. All of the two groups of five vaccinated mice challenged with type 24 streptococci died. Similarly, all five mice vaccinated with strain SL5930 died when challenged with the type 5 strain. In contrast, four of the five mice vaccinated with SL7127, with M5 chimeric flagellin, survived the challenge with the type 5 *S. pyogenes* strain (Table 3).

DISCUSSION

The oligonucleotide used in this investigation comprised 15 codons specifying the 15 N-terminal amino acids of type 5 M protein, together with a terminal CTA codon specifying leucine (to terminate translation of any flagellin genes with the insert in the unwanted orientation). The insert was thus exactly as long as the 48-bp fragment deleted from the flagellin gene in plasmid pLS408, the target for the insertion. The resulting recombinant flagellin gene of plasmid pLS439 caused production of flagellin, which was assembled into functional flagella, as shown by the motility of the flagellin-deficient *E. coli* and *S. dublin* strains given this plasmid; furthermore, the previously nonflagellate *S. dublin* live-vaccine strain given plasmid pLS439 produced normal-looking flagella in about normal numbers, as judged by electron microscopy (Fig. 2). By contrast, in the experiments of Wu et al. (33), in which the inserts used specified hepatitis B surface protein epitopes, three of the six tested plasmids with in-frame inserts in the flagellin gene did not confer motility on the *S. dublin* live-vaccine strain (and this failure was apparently not correlated with discrepancy in length of insert and deletion). However, the three plasmids that did not confer motility did cause production of chimeric flagellin, as shown both by immunoblotting and by the

TABLE 1. Immune response of rabbits to M protein epitope

Immunogen	Rabbit[a]	ELISA titer[b]		Opsonizing activity[c]			
		S-M5(1-15)c	pepM5	Type 5 (Manfredo)	Type 5 (B7788)	Type 5 (Smith)	Type 24 (Vaughn)
S-M5(1-15)c	8801	204,800	12,000	86	54	92	2
	8802	25,600	3,200	62	54	52	0
	8803	51,200	6,400	96	84	96	0
SL7127 (heat killed)	8804	400	200	26	8	8	0
	8805	3,200	6,400	74	44	76	0
	8806	400	400	2	2	2	0
Purified flagella from SL7127	8822	800	800	34	ND	ND	ND
(100 μg per rabbit)	8823	800	1,600	50	ND	ND	ND
	8824	6,400	12,800	76	ND	ND	ND

[a] Rabbits 8801 through 8803 were immunized with 100 μg of synthetic peptide S-M5(1-15) conjugated to keyhole limpet hemocyanin; rabbits 8804 through 8806 were immunized with heat-killed *S. dublin* SL7127, which expresses chimeric flagella; rabbits 8822 through 8824 were immunized with isolated flagella from strain SL7127 at 100 μg per rabbit.

[b] Antibody production is expressed as peak ELISA titers (during 3 months of weekly testing) with S-M5(1-15)c or pepM5 as the coating antigen.

[c] Opsonic antibodies were detected in the sera of rabbits as early as 2 weeks after the initial immunization and peaked at 12 weeks. Values are representative of those obtained at the time of peak ELISA titers. Opsononizing activity is defined as the percentage of neutrophils with associated streptococci after a 45-min rotation in whole heparinized blood containing preimmune or immune serum. Opsonizing activity for preimmune sera of all rabbits was between 0 and 2% for all strains of streptococci. ND, not determined.

production of flagellin-specific and epitope-specific antibodies by animals given the *S. dublin* live-vaccine strain carrying any one of these three plasmids. It thus seems that production of flagellar filaments is not essential for immunization; however, it is convenient, in that it allows the easy collection and purification of the antigenic material. In our strains the expected exposure of the epitope at the surface of the flagellar filaments was shown by immunogold labeling and by the immobilizing effect of antipeptide antibody; we think such surface expression probably results from the location of the site of insertion close to a short segment of the gene for flagellin (of antigenic type *i*) identified as the site of several amino acid substitution mutations causing altered serological specificity (11).

TABLE 2. Immune responses in BALB/c mice immunized with live *S. dublin* SL7127 and SL5930[a]

Strain used for immunization	Week	ELISA titer[b]			Opsonizing activity[c] (%)
		S-M5(1-15)c	pepM5	SL5930	
SL7217	2	200	400	1,600	6
	3	400	1,600	6,400	22
	4	800	1,600	6,400	52
	5	3,200	6,400	25,600	84
	6	3,200	12,800	25,600	96
	8	6,400	12,800	12,800	92
SL5930	2	<100	<100	1,600	0
	3	<100	<100	3,200	0
	4	<100	<100	6,400	0
	5	<100	<100	25,600	0
	6	<100	100	12,800	0
	8	<100	<100	12,800	0

[a] Groups of five mice were immunized with either strain SL7127 or SL5930. Each immunizing and booster dose (at weeks 1, 2, 3, 5, and 6), given intraperitoneally, comprised 1×10^6 to 2×10^6 live bacteria.

[b] Pooled sera from each group of five mice, bled before the first and each subsequent (booster) inoculation, were titrated by ELISA with synthetic peptide S-M5(1-15)c, pepM5, or bacteria of strain SL5930 (no insert in flagellin) as the coating antigen.

[c] Each serum pool was also tested for opsonization of *S. pyogenes* type 5 (strain Smith). Opsonizing activity is defined as the percentage of neutrophils with associated streptococci after a 45-min rotation in whole heparinized blood containing preimmune or immune serum.

Immune responses to both flagellar antigen *d* and the M5 epitope were obtained in all of the rabbits and mice vaccinated by injection of either purified flagella or whole bacteria of strain SL7127 (live or heat or formaldehyde killed). In early bleedings the ELISA titers with flagella as test antigen were higher than those for the M protein peptide; this may reflect the presence of many B-cell epitopes and perhaps helper T-cell epitopes in the exposed part of the flagellin molecule compared with only one or a few in the inserted peptide. However, after a total of five intraperitoneal live-vaccine doses, mice had ELISA titers for peptide S-M5(1-15) about as high as those for flagellar antigen *d*. It would be interesting to see whether an earlier antibody response to the M5 peptide could be elicited by employing chimeric flagellin with multiple M5 oligonucleotide inserts.

It should be noted that we did not test the phagocytosis-promoting activity of sera from immunized mice directly, by testing their bactericidal effect on streptococci mixed with whole blood; instead, we measured the ability of serum samples to cause attachment of type 5 but not type 24 streptococci to neutrophils (see Materials and Methods) (Tables 1 and 2). However, we think it as least very probable that the (partial) type-specific protection against streptococcus challenge seen in mice vaccinated with the *S. dublin* strain that makes chimeric flagellin but not in those given the

TABLE 3. Challenge by intraperitoneal injection of type 5 and 24 streptococci or *S. dublin* SL5608 in BALB/c mice immunized with live *S. dublin* SL7217 or SL5930[a]

Challenge organisms	Survival of mice immunized with strain:		
	SL7217	SL5930	Unimmunized
Type 5 streptococci (Smith strain)	4/5	0/5	ND
Type 24 streptococci (Vaughn strain)	0/5	0/5	ND
S. dublin SL5608	5/5	5/5	0/5

[a] Mice were immunized with 1×10^6 to 2×10^6 viable *S. dublin* SL7127 or SL5930 and then boosted five times with the same dose on weeks 1, 2, 3, 5, and 6. Fifteen days after the last vaccination, mice were challenged with either 1.6×10^6 type streptococci, 2.2×10^5 type 24 streptococci, or 6.3×10^3 *S. dublin*. Data are given as the number of survivors/total. ND, not done.

strain without the M5 epitope in its flagellin reflects the protective effect of opsonic antibody resulting from the immune response to the M5 epitope presented in the flagellar antigen.

The presence of antibody specific for the M5 insert was shown by the ability of both rabbit and mouse sera to immobilize strain SL7127 after complete adsorption with strain SL5930, which is identical except for absence of the insert in the flagellin gene. This immobilization was shown both by effect on spreading growth in semisolid medium and by microscopic observation. This method may prove to be a convenient way to rapidly detect an immune response to an epitope when neither the corresponding synthetic peptide nor the source protein is available for use as test antigen in ELISAs or radioimmune assays, provided that the epitope insertion does not interfere with the assembly of functional flagella.

There is increasing evidence that attenuated strains of *Salmonella* species may have a role as an effective, general vaccine delivery system. It has been demonstrated that these organisms can be used to express a variety of foreign antigens, intact or as peptide epitopes, and carry them to the immune system of the host (20, 22, 23). To optimize this capability, we must continue to explore the molecular process concerning M protein expression such as (i) the location of M gene product(s) within the transformed salmonellae, whether it be cell surface, secreted, periplasmic, or cytoplasmic; and (ii) the quantity, size, and molecular configuration of the cloned immunogens, as they relate to maximizing immunogen delivery for optimum interaction with the B- and T-cell immune system of the host.

ACKNOWLEDGMENTS

The work at Stanford was supported by Public Service Grant AI-27722 (to B.A.D.S.) from the National Institute of Allergy and Infectious Diseases and by a gift from Praxis Biologics Inc. S.N. was a postdoctoral fellow (86/0594-9) of Fundacao de Amparo a Pesquisa do Estâdo de Sâo Paulo, Brazil. The work at Memphis was supported by research funds from the U.S. Veteran's Administration and by Public Health Service grants GM 38530 (to M.K.) and AI-13550 and AI-10085 (to E.H.B.) from the National Institutes of Health.

We thank Edna Chiang, E. H. Beachey's assistant, for excellent technical assistance in the preparation of M protein, antiserum preparation, and mouse and rabbit immunizations and James B. Dale for his input into the mouse protection studies.

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