PROTECTIVE IMMUNITY EVOKED BY ORAL ADMINISTRATION OF ATTENUATED *aroA SALMONELLA TYPHIMURIUM* EXPRESSING CLONED STREPTOCOCCAL M PROTEIN

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The M protein fibrils emanate from the surface of *Streptococcus pyogenes* cells as α-helical coiled coils (1, 2) and render the organisms resistant to ingestion and killing by phagocytic cells in the blood of the non-immune host. In the immune host, type-specific antibodies against the M protein neutralize the antiphagocytic effect and enable the rapid elimination of any invading streptococci bearing the same serotype of M protein (3). Efforts to vaccinate against streptococcal infections have been hampered by toxic reactions to almost any streptococcal product administered to humans (4).

Recent studies have demonstrated protective and autoimmune epitopes within the covalent structures of several M proteins (5–12). In an attempt to overcome toxicity and autoimmune reactions, we investigated the protective immunogenicity of M proteins cloned and expressed in an attenuated *aroA* strain of *Salmonella typhimurium* SL3261, which is able to invade the mucosal surfaces of the intestinal tract but is unable to cause disease (13). We now report that immune responses can be obtained without toxicity by the oral administration of the recombinant *aroA S. typhimurium* vaccine. Our findings have bearing not only on the development of M protein vaccines to protect against streptococcal infections triggering rheumatic fever and rheumatic heart disease, but also on the development of vaccines in general, whereby an attenuated orally administered infectious agent is used as a delivery vehicle to evoke protective immunity against unrelated infectious agents.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. Serotype M5 *S. pyogenes* strains Manfredo and Smith, and M serotype 24 *S. pyogenes* strain Vaughn have been described previously (8, 9). *S. typhimurium* strains SL1344 (*aro*$^+$) and SL3261 (*aroA*$^-$) were a generous gift from Dr. Bruce A. D. Stocker and his associates at Stanford University, Stanford, CA (13). The *S. typhimurium* strain LB5000 (r^-m^+) was kindly provided by Dr. Thomas F. Meyer, Max-Planck-

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Institute, Tubingen (14). The plasmid pMK207, containing the entire genome for the serotype 5 M protein, has been previously characterized by Kehoe et al. (15, 16). SL3261 was transformed with pMK207 (which was obtained from *S. typhimurium* LB5000 harboring pMK207) by a standard procedure (17). All pMK207 harboring bacterial strains ($M5^+$ and Kan^r) were maintained on nutrient agar containing 50 μg/ml kanamycin sulfate. The *aroA*$^-$ SL3261 was maintained on nutrient agar containing 10 μg/ml each of paraminobenzoic acid (PABA)[1] and 2,3-dihydroxybenzoic acid (DHB; Sigma Chemical Co., St. Louis, MO). Polypeptides of type 5 and 24 M proteins were extracted with pepsin and purified as described (8). The extracts are designated pep M5 and pep M24.

Immunization of Mice. BALB/c mice were immunized orally with the pMK207-transformed *Salmonella* SL3261. The organisms were grown as described above for 16 h at 37°C, harvested, washed twice in PBS (0.02 M phosphate, 0.15 NaCl, pH 7.4), and resuspended in sufficient PBS to give a concentration of $\sim 10^9$ CFU per 25 μl. Mice were deprived of water for 24 h and then fed 25-μl doses of the suspension on days 1 and 6. Serum was collected by cardiac puncture at intervals of 1, 3, 5, 9, 10, 15, and 20 wk after immunization. In some experiments, mice were given booster injections intraperitoneally with 50 μg of a pepsin extract (8) of type 5 M protein (pep M5) in 0.1 ml of PBS, 60 h before bleeding.

Antibody Assays. M protein-specific antibodies were measured by ELISA and opsonization assays and were performed as previously described (15). The test mixtures for the opsonophagocytic assays consisted of 25 μl of a standard suspension of streptococci, 25 μl of test serum, and 0.4 ml whole, heparinized (10 U/ml) human blood. After incubation by rotation at 37°C for 45 min, the percentage of neutrophils with associated streptococci (percent opsonization) was estimated by microscopic counts of stained smears (15).

Salivary antibodies were assayed by ELISA using immobilized pep M5 as antigen and serial dilutions of pooled saliva collected at 20 wk. Salivation was induced by injecting each mouse intraperitoneally with 0.1 ml of 2% pilocarpine. In addition to assaying for IgG, the saliva samples were also assayed for the presence of IgA and IgM by using the respective class-specific, goat anti-rabbit antisera (Cappel Laboratories, Cochranville, PA).

Cell Fractionation. *S. typhimurium* SL3261 (pMK207) cells were cultured and fractionated according to the methods described by Fischetti et al. (18). Each cellular compartment was assayed for M protein by Western immunoblot analysis.

Transblot Analyses. Immunoelectrophoresis was performed in order to determine the stability of M5 protein expression in SL3261. The pMK207-expressing bacteria were grown in 10 ml nutrient broth containing 10 μg/ml each of PABA and DHB and 50 μg/ml kanamycin at 37°C for 16 h. The cells were harvested at 8,000 rpm for 15 min and washed thrice with an equal volume of cold PBS in order to remove residual kanamycin. A 0.1-ml aliquot of washed SL3261-pMK207 cells was inoculated into 9.9 ml of fresh nutrient broth containing PABA and DHB but lacking kanamycin, and was incubated at 37°C for 16 h. The subculturing of SL3261-pMK207 (using 1% inoculums) into media lacking kanamycin was repeated four more times. At 16-h intervals, the remaining 9.9 ml of SL3261-pMK207 was pelleted and prepared for immunoblot analysis of M5 protein as previously described (15).

Challenge Infection Experiments. Immunized and control BALB/c mice were challenged intraperitoneally with either the homologous M5 or heterologous M24 streptococci, or with wild-type *S. typhimurium* SL1344. The organisms were grown in Todd-Hewitt broth for 16 h at 37°C, collected, and washed three times in PBS, and finally resuspended to the desired concentration in PBS. 22 d after the initial immunizing dose (see above), each mouse was given intraperitoneal injections of 0.5 ml of bacterial suspension with various concentrations of bacteria. Intranasal challenge doses consisted of 10 μl of bacterial suspension per nostril in lightly anesthetized mice (penthrane). Deaths were recorded over a period of 10 d. All deaths by either route occurred between 2 and 6 d for animals challenged with streptococci and between 5 and 7 d for those challenged with *S. typhimurium*. All surviving mice appeared healthy up to 30 d after challenge.

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[1] *Abbreviations used in this paper:* DHB, 2,3-dihydroxybenzoic acid; PABA, paraminobenzoic acid.

Results

The attenuated *aroA* strain of *S. typhimurium* SL3261 developed by Hoiseth and Stocker (13) was chosen as the delivery vehicle. This mutant strain exhibits nutritional markers both for PABA and DHB (13). The M5 gene (*spm5*) was cloned and expressed in *Escherichia coli* LE392 as described (15, 16). The plasmid pMK207 was isolated, purified, and transformed first into an $r^- m^+$ strain LB5000 of *S. typhimurium* (14, 17). The plasmid isolated and purified from LB5000 was then used to transform *aroA S. typhimurium* SL3261.

Expression of M5 Protein by aroA S. typhimurium SL3261. The transformed LB5000 and SL3261 expressed the entire M5 protein molecule as demonstrated by Western blot analysis of whole cell lysates (Fig. 1, lanes *1* and *2*, respectively). The typical triplet of M5 protein (see References 15 and 16) migrated as bands of M_r 57.9, 55.4, and 52.9 kD. The M5 protein was confined (>90%) to the cytoplasmic compartment as demonstrated by spheroplasting of the transformed organisms. The absence of any M protein on the surface of the organisms was shown by their failure to agglutinate in the presence of antisera previously prepared against pep M5 (9). The M5 protein was expressed in a stable fashion by SL3261 even in the absence of antibiotic pressure during repeated subcultures over 5 d, respresenting ~35 generations of growth (Fig. 1, lanes 3–8). Moreover, SL3261-pMK207 isolated from the liver of a mouse 3 wk after oral inoculation continued to express M5 protein (Fig. 1, lane *9*) (see below).

Mouse Tolerance of Transformed aroA S. typhimurium. BALB/c mice were fed orally with increasing numbers of SL3261-pMK207 to determine the dose of transformed organisms the animals would tolerate. None of the mice receiving up to a maximum of 1.65×10^9 CFU per oral dose either sickened or died. At 1, 3, 5, and 10 wk after inoculation, two mice from each dosage group were killed, and their livers, gall bladders, spleens, and intestines were cultured for SL3261-pMK207 on McConkey's agar containing 50 μg/ml kanamycin sulfate and 10 μg/ml each of PABA and DHB. Non-lactose-fermenting colonies could be isolated only from the mice receiving 10^6 CFU or more at 1 wk and only from those receiving 10^9 CFU of SL3261-pMK207 at 3 wk. No isolates were recovered after 3 wk. The colonies isolated at 3 wk expressed intact M protein (Fig. 1, lane *9*).

Immune Responses in Mice. Based on the results obtained above, groups of mice were immunized orally with 25-μl suspensions of streptococci containing ~10^9 CFU of pMK207-transformed *aroA* organisms on day 1 and 6. Sera obtained at 1, 3, 5,

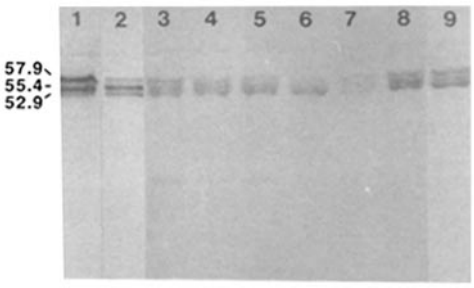

FIGURE 1. Immunoblot analyses of type 5 M protein expressed by pMK207-transformed *S. typhimurium* LB5000 (lane *1*) and SL3261 (lane *2*). Lanes *3-7* demonstrate the expression of M 5 protein by SL3261-pMK207 after repeated subculture in the absence of kanamycin. Lane *8* shows M5 protein expressed by SL3261-pMK207 in the presence of kanamycin using the fifth subculture (lane *7*) as the inoculum. Lane *9* represents M5 protein expressed by SL3261-pMK207 isolated from the liver of BALB/c mice 3 wk after oral immunization.

TABLE I

Immune Responses in BALB/c Mice Orally Immunized with S. typhimurium SL3261-pMK207

Test serum	ELISA titer*			Percent opsonization	
	IgA	IgG	IgM	Type 5	Type 24
Pre-immune	<50	<50	<50	0	2
1 wk	100	200	<50	4	4
3 wk	400	400	<50	36	0
5 wk	800	800	<50	52	2
9 wk	100	1,600	200	90	2
10 wk	50	800	400	92	2
15 wk	100	800	400	52	0
15 wk‡	800	12,800	800	82	0
20 wk	<50	400	200	10	2
20 wk‡	400	3,200	200	80	2
Anti-pep M5§	ND‖	ND	ND	96	2
Anti-pep M24§	ND	ND	ND	0	98

* All mouse antisera that reacted against immobilized pep M24 gave titers of <50.
‡ Mice were boosted 60 h before bleed out with pep M5.
§ Both anti-pep M5 and anti-pep M24 antisera were prepared in rabbits.
‖ The antibody titers for the rabbit anti-pep M5 or anti-pep M24 antisera were not determined.

9, 10, 15, and 20 wk were pooled and examined for antibodies against type 5 M protein and type 5 streptococci by ELISA and opsonization experiments, respectively (Table I). The pooled, pilocarpine-stimulated salivas obtained 20 wk post-immunization contained IgA antibodies against pep M5 in ELISA titers of 1:32 compared with preimmune titers of <1:4 (Table II). 60 h after a booster injection of 50 μg of pep M5, the IgA anti-pep M5 titer was 1:64. There was a twofold increase in antibody titer when saliva was reacted with a mixture of peroxidase labeled anti-mouse IgA + IgG + IgM antisera (Table II). None of the serum or salivary antibodies reacted with cardiac tissues by immunofluorescence tests (9) of frozen sections of human heart or with human heart sarcolemmal membrane polypeptides in Western immunoblots (10, 15).

The absence of reactivity of the above immune sera with heart tissue, in light of previous demonstrations of the presence of such crossreactive antibodies in rabbits immunized with a polypeptide fragment of type 5 M protein (9, 10), prompted the immunization of four BALB/c mice with pep M5 protein (50 μg) emulsified in CFA followed 4 wk later with the same dose in PBS. None of the mice developed heart

TABLE II

Salivary Antibody Response Against M5 Protein in BALB/c Mice Immunized Orally with S. typhimurium SL3261-pMK207

Test serum	ELISA titer against pep M5	
	IgA	IgA + IgG + IgM
Pre-immune	<4	<4
20 wk	32	32
20 wk + boost*	64	128

* Mice were injected with a booster dose of pep M5 60 h before saliva collection.

TABLE III

Challenge by Intra-peritoneal Injection of M Type 5 and Type 24 Streptococci or S. typhimurium SL1344 in BALB/c Mice Immunized Orally with Live aroA S. typhimurium Transformed with pMK207 Expressing Type 5 M Protein

Challenge organisms	Dose*	Survival‡	
		Unimmunized	Immunized
M5 Streptococci (Smith strain)	1.7×10^4	2/4	4/5
	1.7×10^5	0/4	5/5
	1.7×10^6	0/4	5/5
M24 Streptococci (Vaughn strain)	1.6×10^3	1/3	1/3
	1.6×10^4	0/3	0/3
	1.6×10^5	0/3	0/3
S. typhimurium (Strain SL1344)	7.3×10^1	0/3	3/3
	7.3×10^2	0/3	3/3
	7.3×10^3	0/3	3/3

* Dose was determined as the CFU administered.

‡ Survival was recorded as the number of surviving mice divided by the number of mice challenged.

crossreactive antibodies in sera obtained at 8 wk after the initial immunizing dose even though the immune responses were strong as measured by ELISA against pep M5 (ELISA titers = 12,800, 6,400, 6,400, and 3,200, respectively). These results suggest that BALB/c mice may not recognize the autoimmune epitopes of type 5 M protein.

Protective Immunity. Based on these results, a group of mice immunized as above were challenged on day 22 with type 5 or 24 streptococci, or virulent *S. typhimurium* SL1344. The mice inoculated with M5-producing SL3261-pMK207 were completely protected against intraperitoneal challenges of type 5 but not type 24 streptococci

TABLE IV

Challenge by Intra-nasal Inoculation of M Types 5 and 24 Streptococci or S. typhimurium SL 1344 in BALB/c Mice Immunized Orally with aroA S. typhimurium Transformed with pMK207 Expressing Type 5 M Protein

Challenge organisms	Dose*	Survival‡	
		Unimmunized	Immunized
M5 Streptococci (Smith strain)	3.9×10^7	0/4	6/6
M24 Streptococci (Vaughn strain)	3.5×10^7	0/4	0/6
S. typhimurium (strain SL1344)	4.3×10^4	0/4	6/6

* Challenge dose was determined as the CFU administered intranasally and given 13 wk after the initial immunization (i.e., day 1).

‡ Survival was recorded as the number of surviving mice over the number of mice challenged.

(Table III); control mice that were challenged with the virulent *S. typhimurium* SL1344 also survived.

To test the efficacy of the oral vaccine against mucosal infections, a group of orally immunized BALB/c mice were challenged intranasally with type 5 or type 24 streptococci, or with *S. typhimurium* SL1344 (Table IV). Only the SL3261-pMK207-immunized mice survived an intranasal inoculation of type 5 streptococci or virulent *S. typhimurium* SL1344. Protection was M type specific; none of the mice challenged with type 24 streptococci survived. These results indicated that the orally immunized mice were protected against both parenteral and mucosal challenge infections with group A streptococci bearing the same serotype of M protein expressed by the transformed *aroA* strain of *S. typhimurium*.

Discussion

In 1981, Hoiseth and Stocker (13) reported that the introduction of an auxotrophic mutation such as *aroA* into the genome of a virulent strain of *S. typhimurium* rendered the organisms avirulent for mice. Such mutants are phenotypically dependent for growth on several aromatic metabolites, including aromatic amino acids and enterochelin (13). Nevertheless, the organisms retain their ability to colonize and invade the intestinal mucosa before they succumb to the lack of these required nutrients. The short-lived invasion by the *aroA* mutants is sufficient to evoke protective immunity against subsequent challenge infections with the virulent parenteral organisms.

More recently, Brown et al. (19) and Maskell et al. (20) investigated the use of the *aroA* mutant strain of *S. typhimurium* as a vehicle to deliver heterologous non-Salmonella antigens to the immune system in a manner that would stimulate mucosal and humoral immune responses in experimental animals. In this way, antibodies were raised against cloned β-galactosidase by the oral administration of *aroA S. typhimurium* carrying the cloned *lacZ* structural gene (19). Similar immune responses were reported against the K88 antigen and the B fragment of the labile toxin of enterotoxigenic *E. coli* in mice either injected intravenously or fed orally with *aroA S. typhimurium* expressing the respective cloned antigens (20).

We believe our results are the first to show that an antiphagocytic virulence determinant can be cloned and stably expressed in these *aroA* organisms and, further, that the oral administration of the transformed *aroA* mutant can evoke a protective immune response against the unrelated bacterial pathogen. The protective immune response was reflected not only by the survival of the immunized animals challenged with M type 5 streptococci, but also by the development of serum opsonic antibodies and of mucosal M protein-specific IgA. Whether or not the antibodies against M protein in the mucosal secretions had a role in protective immunity was not determined in the present investigation; the endpoint of the in vivo experiments was death and, therefore, the respective contributions of mucosal and humoral immune responses to animal survival was not assessed in any direct way.

We were unable to draw any conclusions concerning the recognition of M protein autoimmune epitopes in the recombinant vaccine administered by the oral route. BALB/c mice immunized with M protein emulsified in CFA by the subcutaneous route also failed to develop crossreactive antibodies, suggesting that this animal species may not be capable of recognizing the autoimmune epitopes of streptococcal M protein.

Interestingly, our cell fractionation studies indicated that most of the M protein produced by the *S. typhimurium* mutant remained in the cytoplasmic compartment even though M protein is secreted into the paraplasmic space when cloned and expressed in *E. coli* (18). Thus, the protective immune response against this particular virulence factor does not require periplasmic or surface expression for recognition by the immune system. Our findings are consistent with those of Brown et al. (19) who successfully produced immune responses to cloned β-galactosidase, a protein known to be confined to the cytoplasmic space.

Plasmid replication and the expression of the M protein encoded by the cloned *spm5* gene appeared to be stable both in vitro and in vivo in the absence of antibiotic pressure. Because of the high degree of stability, this plasmid is a good candidate for use in the design of multivalent vaccines, whereby the *spm5* gene is used as a carrier for the tandem fusion of natural or synthetic oligonucleotides encoding virulence determinants of a number of different M serotypes of group A streptococci or other infectious agents. That tandem hybrid peptides are effective as multivalent protective vaccines has already been demonstrated in our laboratories (11, 12). Thus, our findings provide an approach for the genetic engineering of multivalent M protein vaccines to protect against many serotypes of *S. pyogenes* infections, especially against those strains causing poststreptococcal rheumatic fever and glomerulonephritis.

Summary

Attenuated strains of *Salmonella* have been used effectively as vaccines against typhoid fever. We have investigated the use of such strains to deliver cloned antiphagocytic virulence determinants of unrelated bacteria. The *aroA* strain of *S. typhimurium* SL3261 was transformed with a low-copy plasmid vector pMK207, which contains the cloned gene *spm5* encoding streptococcal M protein, the major virulence factor of these organisms. The transformed SL3261 expressed type 5 M protein in the cytoplasmic fraction, and when fed orally to BALB/c mice, evoked both serum and salivary IgA, IgG, and IgM antibodies directed against type 5 M protein. The orally immunized mice were completely protected against both intranasal and intraperitoneal challenge infections with virulent *S. typhimurium* SL1344 or M5 streptococci. These studies provide evidence that an attenuated strain of *Salmonella* can be used effectively as a general vaccine vehicle to deliver antiphagocytic virulence determinants of unrelated bacteria.

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