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INSERTION OF HETEROLOGOUS EPITOPES IN *SALMONELLA* FLAGELLIN

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INTRODUCTION

Strains of *Salmonella* with non-reverting mutations in the common aromatic biosynthesis pathway have been constructed[4] and found to be attenuated and highly effective as live vaccine in several animal models[12, 14]. The lack of virulence is probably associated with their requirement for paraminobenzoic acid, not available in mammalian tissues. Therefore, aromatic-dependent *Salmonella* multiply for only a few generations in the host; however, they persist, as live bacteria, in the liver and spleen of mice for weeks. Such persistence accounts for a strong immune response by the host, providing long lasting protection against challenge with homologous strains.

Aromatic-dependent *Salmonella* strains have been successfully used to expose heterologous antigens to the immune system. An aromatic-dependent *S.dublin* strain carrying a plasmid harboring the gene for the B subunit of heat-labile *E. coli* enterotoxin was able to evoke serum IgG and mucosal IgA antibodies to LT-B after oral administration to Balb/c mice[2]. Similarly, an aroA strain of *S. typhimurium* carrying a plasmid specifying constitutive expression of beta-galactosidase induced cellular and humoral responses to beta-galactosidase, as measured by footpad swelling test and ELISA[1].

The immune response to a cloned antigen may be enough to cause protection. Indeed, an aroA strain of *S.typhimurium* carrying a plasmid determining production of the M protein of a *Streptococcus pyogenes* strain was able to protect against intraperitoneal

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challenge with 100 LD_{50} *S. pyogenes* after oral immunization of Balb/c mice[11]. Vaccination of Balb/c mice with aroA *Salmonella typhimurium* expressing the circumsporozoite protein from *Plasmodium berghei* conferred protection against rodent malaria[13].

Since the immune reponse directed to some antigens resides in short amino acid sequences (linear epitopes), it is possible to engineer recombinant bacterial proteins carrying foreign antigenic determinants. When expressed by live vaccine strains of *Salmonella*, such hybrid proteins are likely to evoke immune responses to the inserted peptide.

Flagellin, the protein that makes up the bacterial flagellar filament has been considered an attractive target for epitope insertions, since there is great variation in its amfnoacid composition, a fact revealed by the number of flagellar serotypes in nature. The cloning and sequencing of four flagellin genes of different serotypes from *Salmonella* showed near identity between the genes for ca. 150bp at each end of the genes, and increasing diversity toward the central region, where no more than about 30% homology in aminoacid sequence was detected for any pair-wise comparison[16]. A previously identified epitope of flagellar antigen i was located with segment IV[6]. Mutants of a cloned flagellin H1-d gene with altered antigenicity showed a point mutation or deletions within segment IV[9]. Therefore, the central, hypervariable region is thought to specify antigenic determinants present at the flagellar filament's surface. Insertion of epitope-specifying oligonucleotides in that region could result in exposition of a foreign epitope as a flagellar antigenic determinant.

MATERIALS AND METHODS

Plasmid pLS402 is a pBR322 with a 3.8kb insert from *S.muenchen* harboring the H1-d flagellin gene (provided to us by Dr. T.M. Joys). Plasmid pLS408 was derived from pLS402 by cloning the EcoRI insert in pUC19 and deleting a 48bp EcoRV fragment, inside the hypervariable region, generating a single EcoRV site (GAT ATC) in between two codong of the H1-d gene. Oligonucleotides were designed to allow in-frame insertion at the EcoRV site of plasmid pLS408. They were purchased from Operon Biotechnologies Inc., California.

Strain CL447 is a C600-derivative with a deletion at its single flagellin gene, hag[18]. It is non-motile but becomes motile when given plasmids pLS402 or pLS408. Strain LB5000 is an *S. typhimurium* strain r-m+ used to modify plasmids from *E. coli* to prevent restriction in the live vaccine strain SL5928. SL5928 is an *S. dublin* aroA strain with its single flagellin gene replaced by a gene inactived by the insertion of transposon Tn10.

All molecular manipulations were carried out according to Maniatis et al[7]. Protein analysis was made by western-blotting[15] and the immune responses followed by ELISA[3].

RESULTS

Epitope CTP3 of cholera toxin subunit b as insert

The CTP3 epitope comprises residues 50-64 of the B subunit of cholera toxin[5]. Oligonucleotides specifying this sequence were synthesized with a 15bp overlapping region, leaving 5' overhangs to be repaired by Klenow fragment. The resulting double-

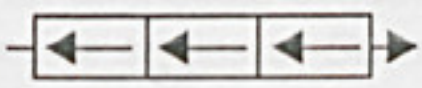
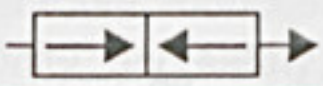
stranded segment was blunt-end ligated to EcoRV-digested pLS408 and transformed into CL447 strain. Plasmids with the insert in correct orientation (as shown by sequencing) were transferred to LB5000 strain and from this strain to SL5928. Hybrid flagellin genes conferred motility on the flagellin-negative recipients (*E. coli* CL447 and *S. dublin* SL5928). Western blotting of lysed bacteria or isolated flagellin showed a single band of the expected size, binding both rabbit anti-d antigen and monoclonal anti-CTP3 antibody. Exposure of CTP3 at the surface of the flagella was shown by immobilization of the bacteria by monoclonal CTP3 antibody and also by immunogold labelling. Live-vaccine strain of *S.dublin* expressing the chimeric flagellin gene was given to C57BL/6 mice, at three weekly intervals, i.p. (5 x 106 bact/mouse) and their sera analysed by ELISA. Sera from all animals reacted with CTP3 peptide and whole cholera toxin [8].

Epitopes of hepatitis B surface antigen as inserts

Epitopes from the surface antigen (S122-137) and from the pre-S2 region (120-145) were inserted in flagellin essentially by the same procedure employed for CTP3 insertion. Results are summarized in table I. Recombinant plasmids with the oligonucleotides inserted in correct orientation (pLS414 for S122-137 and pLS428 for 120-145)

TABLE I

Insertion of oligonucleotides specifying hepatitis B surface epitope (residues 122-137) in vector pLS408. Arrows outside boxes indicate direction of transcription of flagellin gene H1-d. Boxes represent inframe insertions, with the two possible orientations indicated by arrows.

Structure	Plasmid	Motility	Western-blotting	
			anti-peptide	anti-flagellin
	pLS414	-	+	+
	pLS503	+	-	+
	pLS504	+	-	+
	pLS413	+	+	+

originated non-motile *E. coli* or *S. dublin* clones. By western-blotting, a band binding anti-flagellar antigen d and anti-peptide antibodies was detected in both cases, but no flagella were seen by electron microscopy. Insertion of S122-137-specifying oligonucleotide in reverse orientation produced functional flagella, even when 3 copies of the sequence were inserted (48 aminoacids). One recombinant plasmid, pLS413, showed two copies of the oligonucleotides, one of them in correct orientation, and originated functional flagella. Therefore, it was possible to compare the immune response to the hepatitis epitope when present at the flagellar filament's surface (pLS413) or intracellularly, as flagellin (pLS414). Both constructs, when expressed by the live vaccine strain SL5928, evoked antibodies to the inserted peptide to the same level[17].

M protein epitope as insert

The *Streptococcus pyogenes* type-5 M protein comprising the 16 amino terminal residues of the mature protein was inserted in flagellin, since using the whole protein expressed in *Salmonella* to immunize mice p.o. conferred protection against challenge with the *Streptococcus* strain. Immunization with the whole protein, while effective to confer protection, has been hampered by the fact that such protein presents epitopes that cross-react with human heart tissue. The epitope chosen for insertion in flagellin, has been recently characterized as protective, and unable to evoke auto-immune responses[10].

Recombinant plasmids showing the oligonucleotides in correct orientation originated functional flagella, similarly to what has previously been observed for the cholera toxin epitope insertion. Exposition of the epitope at the flagellar filament's surface was revealed by immobilization and immunogold labelling. Mice immunized i.p. with live SL5928 expressing the chimeric flagellin gene and rabbits immunized i.m. with the same strain, formalin-fixed, made antibodies to the M protein peptide, with opsonizing activity (Table II).

Table III summarizes the immune response of all epitopes described herein.

TABLE II

Summary of immune responses of mice vaccinated with strain SL5928 expressing flagellin with an M5 insert or given the same strain expressing flagellin with no heterologous epitopes.

Week	Elisa Titres (*) Peptide	*S. dublin*	Opsonization (**)
Mice immunized with strain SL5928 expressing plasmid pL435 (M5 insert in flagellin)			
0	<100	<100	0
2	200	3,200	6
4	800	12,800	52
6(***)	6,400	25,600	96
Mice immunized with strain SL5928 expressing plasmid pL408 (no insert in flagellin)			
0	<100	<100	0
6(***)	<100	12,800	0

(*) Titres of pooled sera from 5 mice in ELISA test with synthetic M5 peptide or whole bacteria (SL5928) as test antigens.

(**) Percent of human neutrophila with one or more associated streptococci.

(***) Two weeks after the vaccine dose, the mice were challenged by i.p. injection of 100 L.D.50 streptococci. None of the mice given the live-vaccine strain expressing pLS408 survived. Only one of the 5 mice given the hybrid live-vaccine strain died.

TABLE III

Summary of immune responses for all epitopes described in this paper.

Epitope (residues)	Plasmid	Motility	species	doses	no. of doses	route	ELISA (+/tested)
Cholera toxin subunit B (50-64)	pLS411	+	C57BL/6	$5x10^6$, live	x3	i.p.	5/5
			C57BL/6	$5x10^6$, killed	x3	i.p.	5/5
			C57BL/6	10^9, live	x3	p.o.	0/5
Hepatitis B surface protein S (122-137)	pLS414	-	rabbit	10^9, live	x5	i.m.	2/2
			Balb/cJ	$5x10^8$, live	x4	p.o.	10/10
			guinea pigs	10^9, live	x4	p.o.	3/3
Pre-S2 (120-145)	pLS428	-	rabbit	10^9, live	x5	i.m.	2/2
			B10/BR	$5x10^8$, live	x4	p.o.	10/10
			guinea pigs	10^9, live	x4	p.o.	3/3
S (122-137)	pLS413	+	rabbit	10^9, live	x5	i.m.	2/2
			Balb/cJ	$5x10^8$, live	x4	p.o.	10/10
			guinea pigs	10^9, live	x4	p.o.	3/3
S. pyogenes type-5 M protein (42-57)	pLS435	+	rabbit	10^8, killed	x3	i.m.	2/2
			Balb/cJ	$5x10^6$, live	x3	i.m.	5/5
			Balb/cJ	10^9, live	x3	p.o.	0/5

CONCLUSIONS

The central region of flagellin gene H1-d seems appropriate for insertion of epitope-specifying oligonucleotides, since humoral immune responses have been detected against all epitopes inserted so far. The generation of immune responses to the foreign peptide and to flagellin is not dependent upon flagellar function, a fact that greatly extends the probable usefulness of the system. Our results with the hepatitis B surface antigen indicate that it might be possible to insert several epitopes without preventing flagellar assembly and function. However, at the moment we do not know the features of an aminoacid sequence that allow normal flagellar function. Immunization with live-vaccine strains of *Salmonella* presents several advantages: effective immunization by oral route, induction of cellular and humoral immune responses as well as mucosal immunity, and safety, due to the non-reverting aromatic mutation. Experiments are under way to determine the maximum size of insertion compatible with flagellar function, the possibilities of using other sites in the flagellin molecule for insertion of epitopes, and the induction of cellular immunity to a heterologous epitope inserted in flagellin.

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