IgA Response of BALB/c Mice to Orally Administered *Salmonella typhimurium* Flagellin-Displaying T2 Bacteriophages

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DOI 10.1021/bp.132
Published online March 30, 2009 in Wiley InterScience (www.interscience.wiley.com).

Salmonella typhimurium *antigens were displayed on the capsid of a T2 bacteriophage to explore the potential of phage display for an oral vaccine. Segments of the flagellin proteins FliC (H1 antigen) and FljB (H2) were fused to the N-terminal of T2 phage SOC to give two recombinant phages, T2FliCm and T2FljBm. Over 14 days, 19 BALB/c mice were orally administered twice, either with purified recombinant FliCm and FljBm protein, or T2FliCm and T2FljBm with or without host* Escherichia coli. *Feces were sampled over 10 weeks and examined for phage by plaque assay and for the presence of mucosal IgA by ELISA. Relatively few phages were detected relative to the amount administered (up to 8.21 × 10^3 PFU/g faeces) and none were detected five days after initial administration. The administration of a large number of phages appeared to cause no clinical symptoms. IgA concentration in feces peaked around four weeks after the second administration and subsided after eight weeks. The highest relative titers were observed in the protein group (0.37% for anti-FliCm and 0.22% for anti-FljBm) and the mouse group which received no* E. coli *(0.33% and 0.35%) despite the theoretical amount of protein contained in a phage dose being at least 80–465 times lower than the protein dose administered. The possibility that the immunostimulatory properties of the phage create an adjuvant effect to enhance the immunogenic properties of the displayed proteins is discussed. We conclude that phage may be valuable as a vector for oral vaccines.* © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.,* 25: 552–558, 2009
Keywords: bacteriophage display, Salmonella typhimurium*, oral administration, SOC, immune response, flagellin*

Introduction

Bacteriophage (phage) display systems have been used in a wide variety of applications due to the ease of manipulation and unique characteristics of phage such as their specificity and ability to display large fragments fused to capsid proteins.[1] T-even phages such as T4 are good candidates for such systems because they carry nonessential capsid proteins, such as highly immunogenic outer capsid (HOC, 155 copies, 40 kDa) and small outer capsid protein (SOC, 870 copies, 9 kDa), that are easy to manipulate and present at a high copy number.[2,3]

The possibility of displaying antigenic segments from disease-causing organisms to stimulate protective immunity in mammals has been explored using a variety of models. Most trials favor the introduction of the recombinant phage into the blood stream by intraperitoneal injection.[4–6] However, oral administration of recombinant phage in order to achieve immunization remains relatively unexplored, with only one publication to date showing successful immunization, against foot and mouth disease.[7] However, although this study showed that mice can be protected against a virus by oral administration of phage-displayed viral peptides, the immune mechanism leading to this protection was not investigated.

Typhoid fever, caused by the Gram-negative bacterium *Salmonella enterica* serotype Typhi, remains a problem in the developing world, in particular Asia, Africa, and Latin America, where it is estimated to affect 16–33 million people and cause 200,000–600,000 deaths every year.[8,9] Commercial vaccines which are currently available are prohibitively expensive for people in the developing world, require either an injection or multiple oral doses and provide protection to only 50–80% of recipients.[10,11] In addition, the fact that they are based on live, attenuated Ty21a bacteria means they are not suitable for immunocompromised subjects, including those infected with HIV.

Although *S. typhi* does not induce a lethal systemic condition in species other than humans, a model of typhoid fever

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Table 1. Oligonucleotide Primers for PCR

Primer Name	Primer Sequence
fsoc+	AA**CTGCAG**GACTACAAGGACGACGATGACAAGATGGCTAGTACTCGCGGT
mrh2−	TCT**AAGCTT**GGTTTAATCCAACGATTTAACAT
g56+	CG**GAATTC**GAAGAAATCTTTAAACTTTATTATCTG
socN−	CG**GGATCC**TCTCCTTTTATTTAAATTACATGAC
fliCm+	CT**GGATCC**CATGGCCAACGACGGTGAAACTATCGATATC
fliCm−	ACGC**GTCGAC**ATAGTAATCATCGCCTACCTTAACTGC
fljBm+	CT**GGATCC**CATGCTGGACTCACTGAACGTGCAGAAAGCG
fljBm−	AA**CTGCAG**TTTAGTTGCGCCAGCCGCAAGGGTTACTGT
xfliCm+	GA**AGATCT**GGCCAACGACGGTGAAAC
xfliCm−	CCCG**CTCGAG**ATAGTAATCATCGCCTACCT
xfljBm+	GA**AGATCT**CCTGAACGTGCAGAAAGCG
xfljBm−	CCCG**CTCGAG**TTTAGTTGCGCCAGCCGCAA

Restriction sites are in bold.

is provided by *S. typhimurium* which causes a similar disease to typhoid fever in mice. Following ingestion both serotypes infect the organism by transcytosis through M cells and enterocytes in the small intestine mucosa.[12,13] The oral route of infection makes *Salmonella* an ideal target for an oral vaccination which could induce immunity in mucosal membranes that provide the crossing point into the bloodstream. Two of the better-characterized *Salmonella* antigens are the flagellin proteins FliC and FljB, known respectively as the H1 and H2 antigens. Various studies have looked at the possibility of administering these orally to stimulate an immune response.[14–17] These efforts have had some success in producing a humoral immune reaction.

In this study two T2 phages displaying the middle segments of, respectively, the *S. typhimurium* flagellar proteins FliC and FljB linked to the amino-terminal of SOC were constructed by homologous recombination. These recombinant phages were orally administered to mice to ascertain whether they had immunogenic properties.

Materials and Methods

Bacterial strains and bacteriophage

T2β was previously constructed from the well-characterized T2 phage.[18] Briefly, the 3,075 bp β-galactosidase gene of *Escherichia coli* K12 was inserted downstream of the T2 *soc2* and *soc1* genes and upstream of the T2 *soc* gene on a pUC118 plasmid. This plasmid was homologously recombined with T2 phage to give the T2β phage which expresses the enzyme in sufficient quantities to produce blue plaques on bacterial lawns containing X-gal.

The *E. coli* B strain, B^E, was used for bacteriophage propagation and titering. *E. coli* JM109, which lacks the capacity to produce β-galactosidase, was used to screen phages recombined from T2β. *E. coli* BL21(DEα) was used as the host cell for pET plasmids in the IPTG-induced production of recombinant protein.

Construction of plasmids

Plasmids for homologous recombination of phages were constructed from the pUC118 vector (Takara, Japan). The antigenic sequence was inserted upstream of a 379 bp fragment containing the *soc* gene and downstream of a 314 bp fragment containing the *soc2* and *soc1* genes, which have no known function but are necessary for homologous recombination. First the eight amino acid FLAG sequence gene was fused to T2 *soc* by PCR using the *fsoc+/mrh2−* set of primers and inserted into the multi-cloning site (MCS) of pUC118 after PstI/HindIII digestion of both vector and insert (Table 1).

Secondly, the segment containing *soc2* and *soc1* genes was amplified by PCR using the *g56+/socN−* primer set from T2 phage, digested with BamHI/EcoRI and inserted upstream of the *flag* sequence in the BamHI and EcoRI sites of the MCS. Lastly, two gene segments, coding for the amino acids 150–335 of the full-length FliC peptide (the gene is hereafter denoted as *fliCm* and the peptide as FliCm) and amino acids 170–271 of the full-length FljB peptide (referred to as *fljBm* and FljBm), were amplified separately from the *Salmonella enterica* serovar *typhimurium* genome by PCR using the *fliCm+/fliCm−* and *fljBm+/fljBm−* primer sets, digested using BamHI/SalI or BamHI/XhoI and inserted downstream of *soc1* and upstream of *flag* to give plasmids, respectively, named pfliCm and pfljBm. These plasmids were used to transform *E. coli* B^E which was then used as a host for homologous recombination with T2β.

Plasmids for the production of the corresponding recombinant proteins were constructed from pET29d (Invitrogen). The same *fliCm* and *fljBm* segments as described above were amplified by PCR using the *xfliCm+/xfliCm−* and *xfljBm+/xfljBm−* primer sets respectively, digested with BglII/XhoI and cloned into the MCS of pET29d to give the expression plasmids pETfliCm and pETfljBm. *E. coli* BL21(DEα) was transformed using these plasmids.

Recombinant protein expression and purification

To produce recombinant FliCm or FljBm, 30 mL of Luria-Bertani (LB) medium containing 30 μg mL^{-1} kanamycin was inoculated with 300 μL of an overnight culture of pETfliCm-BL21(DEα) or pETfljBm-BL21(DEα) and incubated with 120 rpm agitation at 37°C until reaching early log-phase. IPTG was added to a final concentration of 1 mM and incubated with 120 rpm agitation at 37°C for 4 h. The cultures were centrifuged (10,000*g*, 4°C, 10 min) and the cells were washed before resuspension in 3 mL of 20 mM TrisHCl pH 8.0 buffer. The turbid suspension was sonicated until clear either using a Bioruptor sonicator UCD-200T (Cosmo Bio, Tokyo) or an Ohtake Sonicator 5205 (Ohtake Works, Tokyo).

The debris was precipitated by centrifugation (17,000*g*, 4°C, 10 min) and the supernatant removed to a sterile 15 mL tube.

Concentration and purification of recombinant protein from cell lysate supernatant were performed using a nickel-chelating HisTrap™ column manually operated at a flow rate of approximately 1 mL/min. The column was equilibrated and washed using 10 mM imidazole 20 mM Tris-HCl pH 8.0 and eluted using 60 mM (for FliCm) or 80 mM (for FljBm) imidazole 20 mM Tris-HCl pH 8.0. The eluate was collected in 1 mL fractions. Recombinant protein was visualized by electrophoresis through SDS-polyacrylamide gels which were then stained with Coomassie Brilliant Blue (CBB). Protein concentration was measured using the Bradford Assay in a UV-PharmaSpec 1700 (Shimadzu, Japan).

Phage construction by homologous recombination

T2β is a T2-based phage, which expresses β-galactosidase from its capsid. In a standard plaque assay addition of the substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) to top agar results in the formation of blue plaques due to cleavage by β-galactosidase. Phages expressing FliCm and FljBm respectively were produced by homologous recombination (Figure 1B) between pUCfliCm or pUCfljBm and T2β using the same basic method as described previously.[18] Recombinant phages produce plaques, which are distinguishable by their lack of color, whereas nonrecombined T2β produces blue plaques. The ratio of colorless plaques to blue plaques for both types of plasmid was approximately 1:600. Colorless plaques were picked up under sterile conditions, resuspended in 200 μL SM buffer and incubated at 4°C for at least 6 h to allow phage diffusion. The resulting solution was used in a plaque assay on *E. coli* JM109 and colorless plaques again picked up and put through one more purification cycle. The resulting colorless plaques were assayed by PCR to confirm the presence of the *fliC*m or *fljB*m sequences.

Purification and concentration of recombinant phage

Phages were propagated using the plate lysate method. To purify the phages, the phage lysate was centrifuged through a six-step cesium chloride gradient (1.13, 1.22, 1.29, 1.38, 1.46, 1.55 g/L). CsCl was removed from the fraction containing the phage by dialysis in 1M TrisHCl pH 8.0 then phages were concentrated by ultracentrifugation in a Himac CS100GX (Hitachi) at 10,000*g*, 4°C for 30 min. The pellet was resuspended in 0.25% $CaCO_3$ SM buffer (10 mM $MgSO_4$, 100 mM NaCl, 0.01% gelatin, and 50 mM Tris-HCl [pH = 7.5]) and used for administration.

Oral administration to mice

The recombinant phages were administered orally to 6-week old BALB/c mice (Charles River Japan, Yokohama) using a 1 mL syringe and disposable plastic needle MZ-1 (Clea, Japan). For each administration 100 μL of *E. coli* JM109, mixed T2FliCm and T2FljBm phage, or mixed recombinant FliCm and FljBm protein suspended in 0.25% $CaCO_3$ was injected in the esophagus of the mouse. Twenty-five female mice were divided into four groups labeled A–D as follows: Group A (six naïve mice), no administration; Group B (five mice) administered with purified recombinant 21.8 μg FliCm and 67.1 μg FljBm protein; Group C (seven mice), administered with approximately 10^{10} PFU purified recombinant phage of both types; Group D (seven mice), administered first with approximately 10^9 CFU of *E. coli* JM109 followed by 10^{10} PFU purified T2FliCm and T2FljBm recombinant phages after 3 h. A second administration was undertaken 14 days later using the same procedure, except that the phage number was approximately one order higher at 10^{11} PFU per mouse.

Mice were sacrificed 76 days after initial administration by anesthesia with ether followed by blood extraction from the heart. Blood sera was allowed to separate by simple exposure to air at room temperature for a minimum of 6 h then stored at −20°C until needed. The mice were dissected and examined for pathology.

Fecal sampling and PFU number in feces

Feces were collected and analyzed before administration and afterwards on days 1, 2, 3, 15 (plaque assay) and days 7, 14, 23, 33, 47, 61, and 75 (ELISA). Fresh trays were placed beneath the mouse cages and left for 1 h before collection of feces. Eight to ten of the freshest pellets that were untouched by water, food detritus, or urine were transferred to a 15 mL centrifuge tube (BD Falcon) under sterile conditions. The feces were then either processed directly or stored at −20°C until needed. The feces were weighed and resuspended in phosphate-buffered saline (PBS, pH = 7.4) to a concentration of 50 or 100 mg/mL. The pellets were then homogenized using sterile chopsticks and centrifuged at 1,600*g* for 15 min to remove large fibrous particles. The supernatant was transferred to a fresh tube and centrifuged at 7,200*g* for 10 min, after which the supernatant was used in further experiments.

Enzyme-linked immunosorbent assay (ELISA)

Specific anti-FliCm and anti-FljBm IgA was measured by ELISA using recombinant FliCm or FljBm for coating. Total IgA was measured by sandwich ELISA using an IgA mouse ELISA quantitation kit (Bethyl Laboraties, USA) as specified in the manufacturer's instructions. For both assay types the detection agent was TMB and OD_{450} was measured in a Model 680 Microplate Reader (Biorad, Japan). Anti-FliCm and anti-FljBm IgG titers were measured from blood sera using a similar procedure to that described above for IgA; Total IgG was measured using an IgG mouse ELISA quantitation kit (Bethyl Laboraties, USA) as specified by manufacturer's instructions. Statistical analysis was performed with MS Excel software.

Results and Discussion

Production and purification of recombinant FliCm and FljBm

Recombinant protein was produced from pET-derived plasmids. Figure 1C shows the position in which the cloned *fliCm* and *fljBm* sequences were inserted. *E. coli* BL21(DE3α) was transformed with these plasmids and induced by IPTG to produce soluble proteins which were concentrated in the supernatant of the centrifuged cell lysates. The proteins were then purified and verified by SDS-PAGE (Figure 2). FljBm was 2–3 times more concentrated than FliCm after passage through the nickel-chelating column.

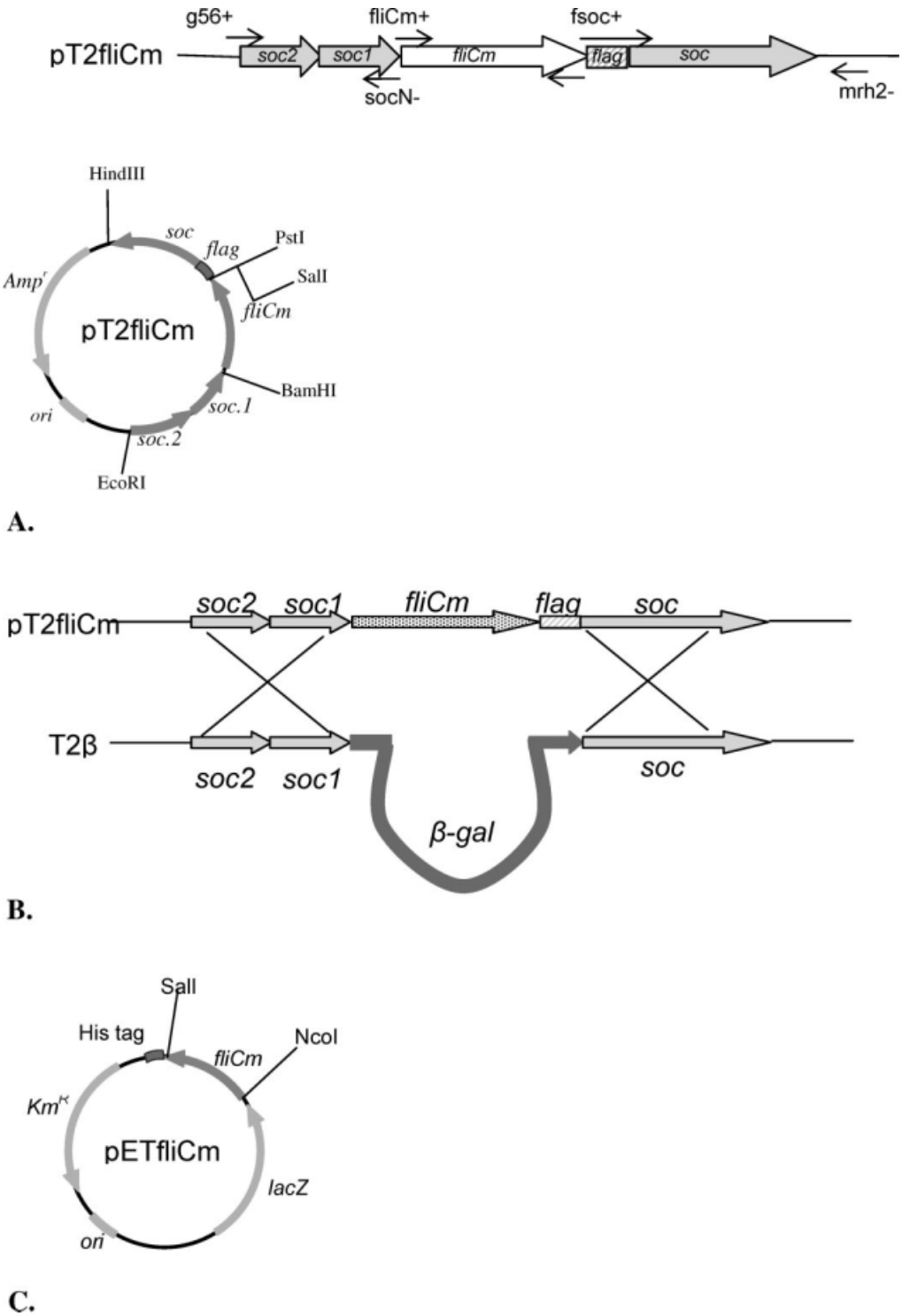

Figure 1. Schematic diagrams of plasmid construction and phage recombination.

(A) Homologous recombination plasmid pT2fliCm with primer binding positions and gene alignment. (B) Homologous recombination between T2β phage genome and pT2fliCm plasmid. (C) Recombinant protein expression plasmid pET fliCm.

Oral administration and phage continuity in intestine

Oral administration of phage or recombinant protein to mice was performed twice interspaced by an interval of two weeks. The administration groups were designated to allow comparison between the effect of the phage in Groups C and D and the effect of protein in Group B and to determine whether the presence of host *E.coli* would increase phage numbers and thus the immunological effect in Group D over Group C. Although the amount of protein was identical in both administrations, mice in Groups C and D were adminstered with around 10^{10} phage particles on the first occasion and approximately 10^{11} on the second.

To evaluate the number of viable phages that survived transit through the digestive system of the mice, plaque assays were performed using the supernatant of fecal samples homogenized in SM buffer to a concentration of 50–100 mg/mL. Fecal pellets vary considerably in water content and texture depending on factors such as the physiological

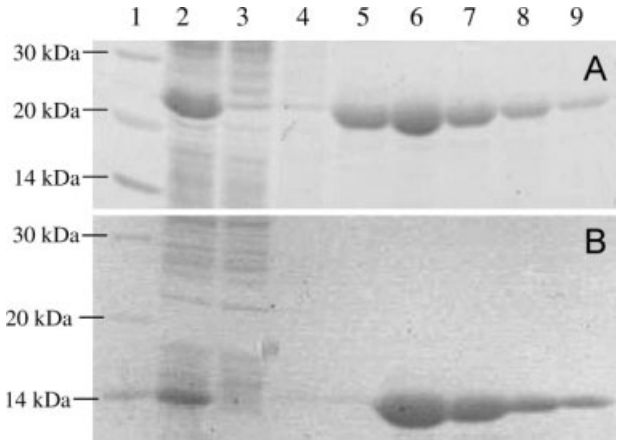

Figure 2. Recombinant FliCm (A) and FljBm (B).

Lanes L-R: 1, molecular marker; 2, cell lysate supernatant; 3, Ni-chelating column flow; 4, column wash; and 5–9, 1 mL fractions eluted in 60 mM imidazole-20 mM Tris-HCl pH 8.0 (FliCm), and 80 mM imidazole-20 mM Tris-HCl pH 8.0 (FljBm).

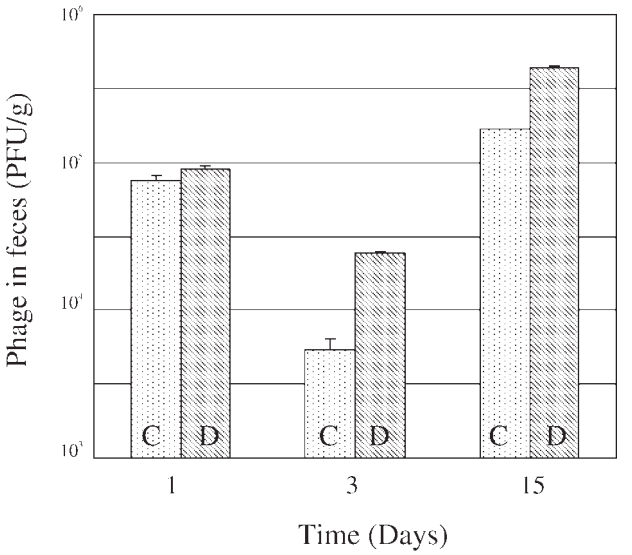

Figure 3. Phage titer expressed as the number of plaque-forming units (PFU) in 1 gm of feces.

Timescale given is days after the first administration; the second administration was performed 14 days after the first. The phage titers are expressed in exponentials of 10 PFU/g feces + SD.

state of the mouse and the freshness of the stool. Therefore, to minimize variation, rigorous conditions were imposed on the sample collection method to ensure obtention of fecal pellets no more than an hour old.

Figure 3 shows the phage titer calculated per gram feces for 1 day and 3 days after the initial administration and 1 day after the second administration (15 days after initial administration). Group D consistently excreted more viable phage (1.42× and 7.05× more one day after first and second administration respectively, increasing to 20.5× more three days after first administration) than Group C which suggests that the phage infected the host *E. coli* in the gut and proliferated successfully. There was considerable individual variation between the mice, as big as 1–2 orders of magnitude in some cases. No phages were detected in feces of either group after 5 days following administration. The greater numbers of phages excreted after the second administration is consistent with the approximately one order greater dose administered relative to the first one.

The results indicated that the majority of the phages succumbed at some point in the digestive process. Precise calculation is difficult as murine fecal output is greatly variable but it seems that as little as one in 10^3 or 10^4 phages administered is passed in feces, which implies massive degradation of phage particles. The suspension of phage in $CaCO_3$ prior to administration has been shown to insulate phage from acidity as strong as pH = 2.0, a similar level to that of the stomach, but the phage may also be vulnerable to other biochemical factors such as hepatic and pancreatic enzyme activity.[18] Although coadministration of host *E. coli* cells for T2 had some effect on the number of viable phages excreted, it did not have a significant effect on the persistence of phage in the intestine. It has previously been reported that phage can persist in feces for as many as nine days after a single oral administration, but that was not observed here.[18]

One concern for vaccine vectors is toxicity. However, administration of large numbers of phages to the mice produced no visible pathology, either during the period of the trial or upon dissection after sacrifice. This supports other studies where T-even phage has been administered to humans or mice in large quantities.[7,18,19]

Quantification of IgA in feces

The predominant immunoglobin class in the mucosal epithelium of the intestine is IgA. It was therefore speculated that production of anti-FliCm or anti-FljBm IgA would be a desirable part of a successful vaccination strategy that would intercept *Salmonella* before it had a chance to enter the blood stream through the intestinal wall. The FliCm and FljBm sequences were chosen because they are known to contain a number of epitopes.[20,21] IgA was measured by diluting the supernatant of fecal solution described above and using it in an ELISA where the coating agent was either recombinant FliCm or FljBm. In light of the variability in water content and texture of feces, the total amount of IgA was measured by sandwich ELISA and the FliCm- and FljBm-specific IgA was calculated as a proportion of the total. This was regarded as being a more accurate measure of antibody production than merely measuring the optical density in an ELISA.

An analysis of the evolution of specific IgA presence in feces showed that the response was slow to develop (Figure 4). No significant response was observed relative to samples from naïve mouse until Day 33. Both anti-FliCm and anti-FljBm IgA concentrations peaked around 4 weeks after the second administration (47 days after initial administration) and had largely subsided after 6 weeks (61 days). No specific IgA was detected after 8 weeks (data not shown). ELISAs conducted using sera of the mice were unable to detect any IgG specific to FliCm or FljBm, indicating that the antigenic particles (protein or phage) failed to enter the bloodstream in sufficient quantities to trigger an immune response.

The highest proportion of specific IgA was observed in Group B (0.373%) for anti-FliCm and in Group C (0.346%) for anti-FljBm. However, there was a large variation between individual mice in a given group, with some showing no response at all. Group B (recombinant protein) in particular showed a large disparity between three mice with no reaction, one with a moderate reaction and one that had a

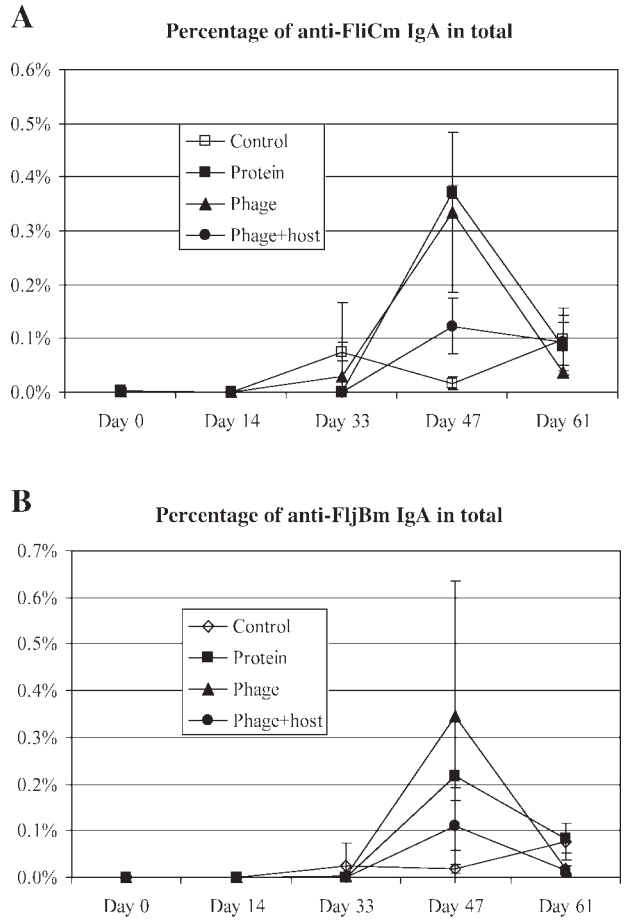

Figure 4. Anti-FliCm (A) and anti-FljBm (B) IgA as a percentage of total IgA detected in feces 46 days and 61 days after initial administration.

White lozenges or squares (◇, □) represent Group A values (naïve mice), black squares (■) represent Group B (recombinant protein) values. Black triangles (▲) represent Group C (recombinant phage) values and black circles (●) represent Group D (recombinant phage + *E. coli*) values. Values are expressed as a percentage of total IgA ± SD.

huge reaction. This contrasted with the mice in Group C (phage only), which all showed similar proportions of anti-FliCm and anti-FljBm specific IgA, and those in Group D (phage and *E. coli*) of which only one showed no response. Statistical treatment of the data showed that the peak responses of Group C and Group D mice were significant relative to the control group ($P < 0.01$ for Group C, $P < 0.03$ for Group D) whereas those of Group B mice were not ($P = 0.24$ for FliCm and $P = 0.34$ for FljBm).

Despite the higher numbers of PFU in feces, Group D developed a smaller immune reaction as quantified by both IgA species (0.123% for anti-FliCm and 0.111% for anti-FljBm). However, although the phage titer between the groups differed more greatly as time went on, there was less of a disparity in the 24 h following administration. This would appear to indicate that the amount of phage reproduction in Group D and resulting greater numbers of phages was insufficient to influence results, and that in this case the peak amount of antigen delivered was a more important factor in triggering a humoral immune response in the intestinal mucosa.

Calculation of the theoretical amount of protein displayed on the T2 capsid suggests that even if FliCm were perfectly fused to each SOC molecule and correctly displayed on the capsid, 100 μL recombinant protein solution administered to Group B contains roughly 80 times more protein than the same volume of T2FliCm phage administered to Groups C and D. The greater concentration of recombinant FljBm makes the disparity even greater relative to T2FljBm administration (465 times). Furthermore, although theoretically phage display could bind a protein to each of the 870 copies of SOC, published analyses of similar T2 and T4 phage display systems have found the actual number fused to the phage capsid to be as little as 10–40 full-length molecules.[22,23] This suggests that the number of protein molecules contained in the recombinant phage doses was another two orders of magnitude smaller relative to the recombinant protein doses. Despite this, administration of phage stimulated a comparable immune response to that engendered by purified protein (0.334% vs. 0.373% for anti-FliCm and 0.348% vs. 0.218% for anti-FljBm), suggesting that the antigenic effect is somehow amplified by proximity to the phage. Possible explanations include the grouping of protein sequences in a regular arrangement at high density, whereas recombinant protein may be sparsely spread, or the putative action of the phage vector, which is immunogenic in itself, as an adjuvant. Another possible reason for the relatively high performance of recombinant phage could be that more intact particles reach the intestine than recombinant protein, either because phage is more resistant to digestive processes or because it has the capacity to reproduce and thus survive longer. However, as the recombinant protein in feces was not measured this remains speculation. The mechanism by which a peptide displayed on a phage particle produces a greater immune response than a greater quantity of the same peptide in free form requires further investigation. This would be of particular value if phage particles were shown to have an inherent adjuvant quality.

One limitation of this study is that the mice were not challenged in order to ascertain whether the immune response induced was sufficient to protect the mice from *Salmonella* following vaccination. Without such data it cannot be said whether the mice were successfully vaccinated or not. We intend to address this issue in our future work.

Given the impact of such a small quantity of phage, it is tempting to speculate that if more viable phage reached the intestine, a greater immune response would be observed. Two possible ways to do this would be to better protect the phage upon administration or to increase phage propagation in the large intestine. The former could be realized by administering the phage in a buffer containing agents to neutralize aggressive digestive enzymes or in a degradable capsule that would disintegrate by the time the phage reached the intestine. The latter could be achieved by using a phage which has a broader host range among the intestinal flora. When compared with T2, a number of phages isolated from sewage influent have a wider host range among *E. coli* strains.[24] If sufficient phage reproduction could be achieved, even a small amount of phage would suffice to trigger an immune reaction capable of providing protection from an invading pathogen.

Conclusion

Two recombinant phages, T2FliCm and T2FljBm, were created by homologous recombination of a T2 derivative, T2β, with a plasmid containing the middle sequences of

either of the *S. typhimurium* flagellin genes *fliC* and *fljB*. The phages displayed the corresponding peptides on their capsids by linkage to SOC. The phages were administered to BALB/c mice with and without host *E. coli* through the oral route and the mice's feces were then assayed for phage and IgA. Viable phages were detected in the feces until five days after administration at higher titers in the group where *E. coli* was administered, indicating that at least some phage reproduction occurred in the digestive system. However, only a small proportion of phage administered survived intact to be passed in feces.

The ELISA results showed that all groups produced specific anti-FliCm and anti-FljBm IgA, with the response peaking four weeks after the second administration. The most IgA was produced by the group that was administered protein (anti-FliCm IgA made up 0.373% and anti-FljBm IgA 0.218% of total IgA) and the group that was administered phage without *E. coli* (0.334% and 0.348%, respectively). Despite the fact that up to 465 times less protein was displayed by the phages than was contained in the recombinant protein dose, the similarity in the degree of immune response suggests that phage display increases the immunogenicity of the displayed protein by an unknown mechanism, possibly by acting as an adjuvant. This research shows the potential of using bacteriophage as a vector for orally administrated vaccines.

Acknowledgments

The authors are grateful to Mikiko Endo for assistance during the *in vivo* experiments. They also thank Dr. Shuji Kanamaru for advice on centrifugation procedures.

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Manuscript received July 28, 2008, and revision received Aug. 22, 2008.