INFECTION AND IMMUNITY, May 1997, p. 1926–1930 Vol. 65, No. 5
0019-9567/97/$04.00+0

NOTES

Expression of the *Yersinia pestis* Capsular Antigen (F1 Antigen) on the Surface of an *aroA* Mutant of *Salmonella typhimurium* Induces High Levels of Protection against Plague

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Received 13 August 1996/Returned for modification 30 September 1996/Accepted 30 December 1996

The *caf* operon from *Yersinia pestis* encoding the structural subunit (*caf1*), the molecular chaperone (*caf1M*), the outer membrane anchor (*caf1A*), and the regulatory protein (*caf1R*) was cloned into *Salmonella typhimurium* SL3261 *aroA*. The recombinant *Salmonella* organisms were encapsulated when cultured at 37°C but not when cultured at 28°C. Oral inoculation of mice with the recombinant *Salmonella* induced predominantly an immunoglobulin G2a response to F1 antigen, and isolated T cells showed a recall response to soluble or *Salmonella*-associated F1 antigen. Mice immunized with *S. typhimurium* SL3261 *aroA* expressing F1 antigen intracellularly developed lower antibody responses to F1 antigen and showed a T-cell recall response only to *Salmonella*-associated F1 antigen. Mice immunized orally with two doses of the recombinant *Salmonella* which expressed F1 antigen on the surface were protected against 10^7 50% lethal doses (LD_{50}) of virulent *Y. pestis* given by the subcutaneous route of challenge, whereas mice immunized with the recombinant *Salmonella* expressing F1 antigen intracellularly were only partially protected against 10^5 LD_{50} of *Y. pestis*.

Although *Yersinia pestis* no longer causes pandemics, the bacterium is still a public-health problem on the Asian subcontinent and in the Far East (4). Most cases of disease caused by *Y. pestis* are of the bubonic form, arising following a bite from a flea which has previously fed on the blood of an infected rodent (4). Some cases of bubonic plague progress to the pneumonic form, which can be transmitted from human to human, leading to epidemics (8). Currently available vaccines which contain heat- or formaldehyde-killed bacteria (19) induce protection against the bubonic form of the disease (23) but are reactogenic (18). Immunity to infection has been correlated with the presence of antibody to the capsular F1 antigen (30), and immunization with the F1 antigen induces protection against the disease in animal models (20, 25, 32). These vaccines require repeated immunizations to evoke protective immunity and are defeated by a high-level challenge (1, 23, 32). The F1 capsule structural subunit is encoded by the *caf1* gene (12), and we previously reported that immunization with *Salmonella typhimurium* which expressed the *caf1* gene and accumulated F1 antigen intracellularly resulted in the induction of a protective response against *Y. pestis* (22). However, the recombinant plasmid was retained in *S. typhimurium* only when mice were codosed with ampicillin. Export and assembly of the F1 capsule are directed by the *caf1A* (17) and the *caf1M* (13) gene products. These genes form an operon including the *caf1* gene, which is regulated by the *caf1R* gene product (16). We have investigated the possibility that expression of the *caf* operon in *S. typhimurium* would result in stable high-level expression of F1 antigen on the surface of the bacterium and would provide enhanced immunity against *Y. pestis.*

Construction of *S. typhimurium* containing the *caf* operon. Two DNA fragments, which together included the *Y. pestis caf* operon (16, 17), were amplified by PCR (35 amplification cycles; 95°C for 15 s, 50°C for 15 s, 72°C for 30 s) (Perkin-Elmer 9600 GeneAmp PCR system) with *Y. pestis* MP6 template DNA (22) and oligonucleotide primer pair FIOU2 (5′TG CCCCGGGAAT TCCGAACATAAATCGG TTCAGT GGC C3′) and M4D (5′GGCGTATTCCTCGCTAGCAATGTTTA ACG3′) or M3U (5′ATCGTTAAACATTGCTAGCGAGGA ATACGCC3′) and FIOD2 (5′GATAGATCTGTCGACTGA ACCTATTATATTGCTTCGCGC3′). The amplified DNA fragments were ligated at the unique *Nhe*I site within the *caf* operon and the DNA was cloned into a low-copy-number plasmid (pLG339; Kan [28]) to generate plasmid pAH34L. After nucleotide sequencing (24) of the *caf1* open reading frame, the deduced amino acid sequence of the *caf1* gene from strain MP6 was shown to be identical to the previously reported sequence from strain EV (12).

The *caf* operon, expressed in *S. typhimurium*, results in the formation of encapsulated bacteria. Plasmid pAH34L was passaged through *S. typhimurium* LB5010 to methylate the DNA and was then electroporated into *S. typhimurium* SL3261. Transformants were unable to grow on minimal media without supplements of aromatic amino acids, confirming their auxotrophic phenotype. Sonicates (22) of *S. typhimurium* SL3261/pAH34L contained 12.8 μg of F1 antigen/10^8 cells when an enzyme-linked immunosorbent assay was performed with purified F1 antigen from *Y. pestis* (32) as the standard antigen. The reported molecular mass of the F1 antigen is 15.5 kDa (12), and in Western blots (Fig. 1) a band with an M_r of 16,000, which reacted with an F1 polyclonal antibody (32), was identified in lysates of *S. typhimurium* SL3261/pAH34L cultured for 18 h at 37°C in L broth containing kanamycin (50 μg ml^{-1}). Cell extracts which were not boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer prior to analysis contained additional antibody-reactive bands with M_rs of 32,000, 49,000, 64,000, and 79,000, indicating oligomerization of F1 antigen. Antibody-reactive bands were not iden-

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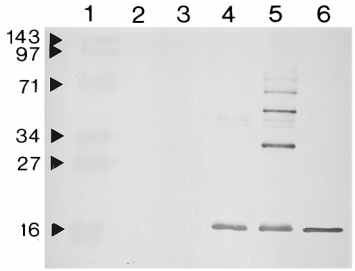

FIG. 1. SDS-PAGE (12% gels) and Western blotting using a murine polyclonal F1 antibody. Blots were developed with a gold-labelled goat anti-mouse antibody (Amersham) with silver enhancement. Samples analyzed (20 μl) were prepared from 8×10^9 cells ml^{-1} of *S. typhimurium* SL3261 cultured at 37°C (lane 2) and *S. typhimurium* SL3261/pAH34L cultured at 28°C (lane 3) or at 37°C (lane 4), all of which were boiled with an equal volume of SDS-PAGE buffer before analysis. Lane 5 contained *S. typhimurium* SL3261/pAH34L cell lysate which had not been boiled with SDS-PAGE buffer immediately before analysis. Lane 1 contained molecular weight markers (numbers on the left are in thousands), and lane 6 contained 22 μg of purified F1 antigen from *Y. pestis* which had been boiled with SDS-PAGE buffer before analysis.

tified in lysates of *S. typhimurium* SL3261 cultured in L broth (Fig. 1). In *Y. pestis*, F1 capsule biosynthesis is temperature regulated (10, 16), and F1 antigen production was not detected in cell lysates of *S. typhimurium* SL3261/pAH34L which had been cultured at 28°C, confirming that the *caf1R* gene was functional in *S. typhimurium* SL3261.

The encapsulated appearance of *S. typhimurium* SL3261/pAH34L (Fig. 2) after films were stained with India ink (7) was similar to the reported appearance of *Y. pestis* after similar staining (3). Confirmation of the surface location of the F1 antigen was obtained when cells of *S. typhimurium* SL3261/pAH34L fluoresced during an immunofluorescent assay, whereas cells of *S. typhimurium* SL3261/pFGAL2a (which expresses the F1 antigen intracellularly [22]) did not fluoresce (Fig. 3).

Plasmid stability in vitro and in vivo. Previous workers have found difficulty in achieving stable high-level expression of heterologous antigens in *Salmonella aroA* (5, 6, 9, 11, 22). The retention of pAH34L by *S. typhimurium* SL3261 was assessed by culturing bacteria in L broth without antibiotic at 28 or 37°C and plating dilutions onto agar with (for the selection of bacterium-containing plasmids) or without kanamycin. Kanamycin resistance was retained by 85 or 29% of the bacteria after culture for 24 or 72 h, respectively, at 37°C. When bacteria were cultured in broth at 28°C for 72 h, all of the bacteria remained kanamycin resistant. This finding indicated that plasmid pAH34L could be segregated efficiently and that plasmid instability in vitro resulted from expression of the *caf* operon.

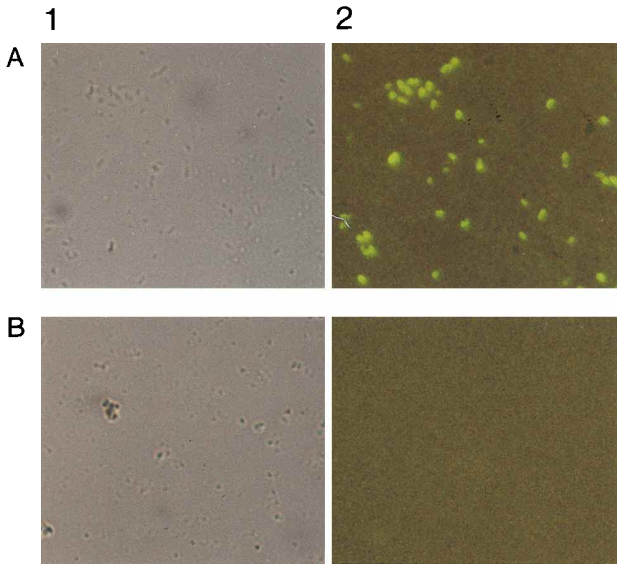

FIG. 3. Phase-contrast microscopy (panel 1) and immunofluorescence (panel 2) of bacteria which had adhered to polytetrafluoroethylene-coated glass slides (Hendley-Essex). The slides were incubated for 1 h at 37°C with murine polyclonal antibody against purified *Y. pestis* F1 antigen (30) (diluted 1:1,000 in 2% BLOTTO in PBS [pH 7.2]) and were rinsed three times in PBS (pH 7.2). After incubation for 1 h at 37°C with fluorescein isothiocyanate-labelled goat anti-mouse antibody diluted 1:100 in PBS (pH 7.2) and rinsing of the slide in PBS (pH 7.2), bacteria were visualized with a fluorescence microscope (Leitz Dialux 20). (A) *S. typhimurium* SL3261/pAH34L; (B) *S. typhimurium* SL3261/pFGAL2a, which expresses the F1 antigen intracellularly (22).

The in vivo stability of plasmid pAH34L in *S. typhimurium* and the tissue-colonizing ability of the bacteria were determined after inoculating groups of six female 6-week-old BALB/c mice (Charles River Laboratories, Margate, United Kingdom) intravenously (i.v.) or intragastrically (i.g.) by gavage. The bacteria isolated from spleens 7 days after inoculation were all kanamycin resistant. The numbers of *S. typhimurium* SL3261 and *S. typhimurium* SL3261/pAH34L organisms recovered from spleens were similar (Table 1), indicating that the F1 antigen did not mask the surface antigens required for invasion of host cells. The stability of pAH34L after immunization of the mice, compared with the instability of the plasmid in vitro, suggests that the surface-expressed F1 antigen confers a selective advantage on *S. typhimurium* SL3261 in vivo.

All of these findings suggest that it will be possible to achieve stable high-level expression of F1 antigen in strains of *S. typhi*

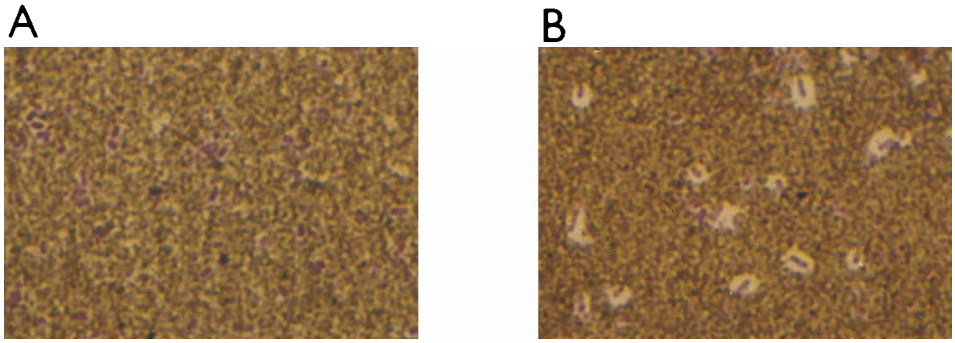

FIG. 2. *S. typhimurium* SL3261 (A) and *S. typhimurium* SL3261/pAH34L (B) cells photographed by light microscopy after staining with India ink.

TABLE 1. Colonization of spleens after i.g. and i.v. dosing[a]

Construct (route)	CFU/spleen enumerated on[b]:		% of organisms retaining plasmid
	L agar	L agar + kanamycin	
S. typhimurium SL3261/pAH34L (i.g.)	$(9.51 \pm 2.3) \times 10^3$	$(9.49 \pm 2.1) \times 10^3$	100
S. typhimurium SL3261 (i.g.)	$(5.49 \pm 3.12) \times 10^3$	NA[c]	NA
S. typhimurium SL3261/pAH34L (i.v.)	$(2.01 \pm 0.84) \times 10^5$	$(2.02 \pm 0.83) \times 10^5$	100
S. typhimurium SL3261 (i.v.)	$(2.27 \pm 0.2) \times 10^5$	NA	NA

[a] Inoculation i.g. with 2×10^8 CFU of *S. typhimurium* SL3261 or 5×10^8 CFU of *S. typhimurium* SL3261/pAH34L or i.v. with 6×10^4 CFU of *S. typhimurium* SL3261 or 5×10^5 CFU of *S. typhimurium* SL3261/pAH34L).
[b] Means derived from six animals in each group ± standard deviations.
[c] NA, not applicable.

which might be used as vaccine vectors in humans. Since we have achieved high-level expression by using a low-copy-number vector, chromosomal integration of the *caf* operon should allow high-level expression of F1 antigen.

Surface-expressed F1 antigen induces serum antibody responses and cell-mediated immunity responses to F1 antigen. Groups of 24 mice were immunized twice (at days 1 and 21) i.g. with 2×10^8 to 5×10^8 CFU of *S. typhimurium* SL3261 or *S. typhimurium* SL3261/pAH34L in 100 μl of phosphate-buffered saline (PBS). A further group of 24 mice was immunized with *S. typhimurium* SL3261/pFGAL2a according to the same protocol but was dosed with ampicillin (22) to promote retention of the plasmid. Antibody against the F1 antigen was not detected in mice immunized with *S. typhimurium* (Fig. 4). Animals immunized with *S. typhimurium* SL3261/pAH34L showed detectable levels of F1-specific immunoglobulin G (IgG) in serum from day 16, and by day 52 titers of F1-specific IgG in serum were high, with the concentration of F1-specific IgG2a in serum exceeding that of IgG1 by more than 30-fold (384 ± 32 μg ml^{-1}, compared with 12 ± 4 μg ml^{-1}). Mice which had been immunized with *S. typhimurium* SL3261/pFGAL2a showed a high level of serum antibody to F1 antigen at day 44 which had declined by day 52 to 6 ± 1.5 μg of IgG2a ml^{-1} and 1.8 ± 0.2 μg of IgG1 ml^{-1}. The failure of *S. typhimurium* SL3261/pFGAL2a to induce a sustained antibody response may be due to the instability of the plasmid encoding the F1 antigen in *S. typhimurium* in spite of the ampicillin dosing of mice.

The production of IgG2a in the mouse is associated with a cytokine profile that is indicative of a Th1 cell response (21, 27), and the predominant IgG2a response in serum suggests that a Th1 cell response was induced by surface-expressed F1 antigen. The ability of *Salmonella*-delivered vaccine antigens to induce predominantly Th1 cell responses has been reported by other workers (2, 29).

The in vitro recall response of T cells isolated and purified (32) from the spleens of six animals in each treatment group to *Salmonella*-presented or to purified F1 antigen was measured

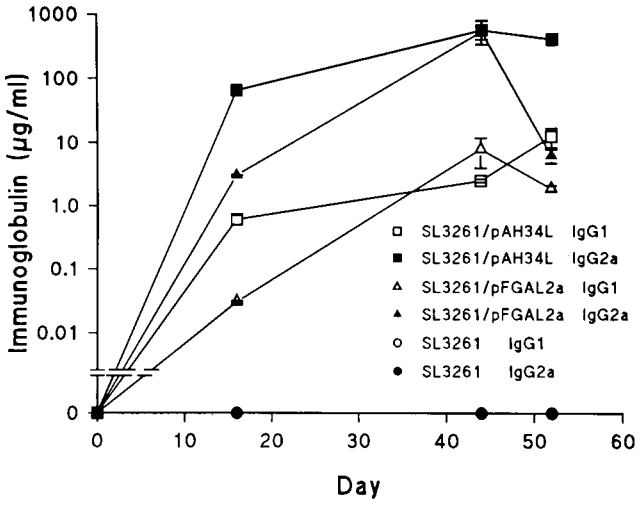

FIG. 4. Determination of levels of F1 antibody in serum collected from immunized mice at days 16 to 52 of the experiment. The measurement of IgG subclass was performed on individual samples. Briefly, samples were double diluted on microtiter plates precoated with F1 antigen (5 μg ml^{-1} in PBS). Bound antibody was detected with peroxidase-labelled secondary antibodies against mouse IgG subclasses (Harlan-Sera Lab, Crawley Down, United Kingdom), and for each sample the titer was recorded as the maximum dilution of sample giving an absorbance at 414 nm of 0.1 unit over the background. Quantification of IgG subclasses in diluted samples (14) was accomplished by converting the absorbance values at 414 nm to nanograms of IgG per milliliter by reference to individual standard curves constructed for each IgG subclass protein (range, 1 to 50 ng ml^{-1}) with the homologous secondary antibody conjugate. From this, mean values (micrograms per milliliter) ± standard errors of the means for IgG1 and IgG2a in serum were derived for each immunization group.

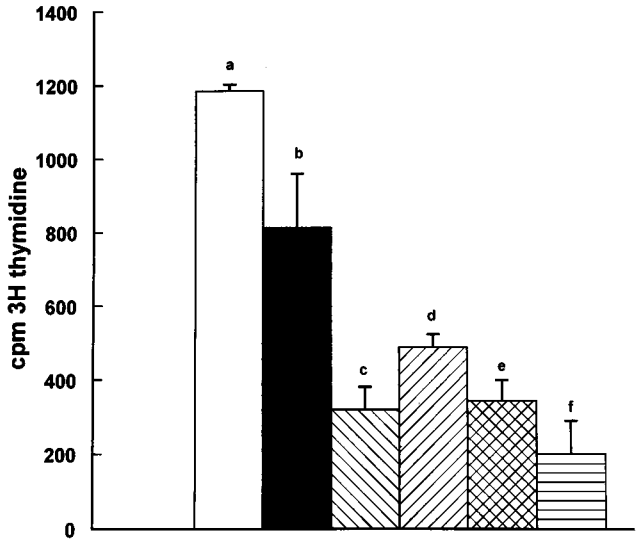

FIG. 5. The in vitro recall response of T cells derived on day 52 from mice immunized with *S. typhimurium* SL3261/pAH34L (bars a and d), *S. typhimurium* SL3261/pFGAL2a (bars b and e), and *S. typhimurium* SL3261 (bars c and f) to a whole-cell lysate of *S. typhimurium* SL3261/pAH34L containing 0.2 ng of F1 antigen ml^{-1} (bars a through c) or 50 μg of soluble purified F1 antigen ml^{-1} (bars d through f). Incorporation of ^3H is shown as values above the background levels found when *S. typhimurium* SL3261/pAH34L, *S. typhimurium* SL3261/pFGAL2a, and *S. typhimurium* SL3261 T cells were incubated with medium only (mean = 135 cpm).

TABLE 2. Survival of control mice and mice previously immunized with *S. typhimurium* SL3261, *S. typhimurium* SL3261/pAH34L, or *S. typhimurium* SL3261/pFGAL2a after challenge with *Y. pestis* GB

Immunization	Challenge dose (CFU of *Y. pestis*)	No. of mice surviving[a]	Mean time to death ± SEM (days)
None	1.2×10^5	0	3.7 ± 0.3
	1.2×10^7	0	3.2 ± 0.2
S. typhimurium SL3261	1.2×10^5	0	5.2 ± 0.5
	1.2×10^7	0	3.7 ± 0.2
S. typhimurium SL3261/pAH34L	1.2×10^5	6	NA[b]
	1.2×10^7	6	NA
S. typhimurium SL3261/pFGAL2a	1.2×10^5	3	7.7 ± 1.7
	1.2×10^7	2	6.5 ± 0.4

[a] Each group had six mice. Deaths were recorded up to 14 days postchallenge.
[b] NA, not applicable.

at day 52 (Fig. 5). A suspension of T cells (100 μl at 10^6 ml^{-1}) was introduced into the wells of a microtiter plate, and proliferation was measured, with three replicates, as [^{3}H]thymidine incorporation into cells. When F1 antigen in solution (6.3 to 200 μg ml^{-1}) was used to stimulate T cells, 50 μg of soluble F1 antigen ml^{-1} was required for the maximum response. At this concentration of F1 antigen, the proliferative response of T cells isolated from mice immunized with *S. typhimurium* SL3261/pAH34L (Fig. 5, bar d) was significantly greater ($P < 0.05$) than the response of T cells isolated from mice immunized with *S. typhimurium* SL3261/pFGAL2a (Fig. 5, bar e) or with *S. typhimurium* SL3261 (Fig. 5, bar f).

When dilutions of a lysate of *S. typhimurium* SL3261/pAH34L (10^{10} CFU ml^{-1}) containing 0.04 to 12.3 ng of F1 antigen ml^{-1} were tested, much lower levels of F1 antigen (0.2 ng ml^{-1}) were required to promote the maximum proliferation of T cells. This suggests that the T-cell recall response was provoked by epitopes in both F1 antigen and *Salmonella*. When a lysate containing 0.2 ng of F1 antigen ml^{-1} was used, the proliferative response of T cells from mice immunized with *S. typhimurium* SL3261/pAH34L (Fig. 5, bar a) was found to be significantly higher ($P < 0.001$) than the response of T cells from mice immunized with *S. typhimurium* SL3261 (Fig. 5, bar c).

The ability of the surface-expressed F1 antigen to induce humoral and cellular responses which cross-reacted with F1 antigen in solution indicated that association of F1 antigen with *Salmonella* is not a requirement for a recall immune response. In contrast, T cells from mice immunized with *S. typhimurium* SL3261/pFGAL2a, unlike T cells from mice immunized with *S. typhimurium* SL3261, proliferated significantly ($P < 0.05$) when exposed to 0.2 ng of F1 antigen associated with *Salmonella* ml^{-1}, but they did not proliferate significantly to 50 μg of soluble F1 antigen ml^{-1}, indicating the absence of a memory T-cell response to soluble F1 antigen.

Immunization with *S. typhimurium* expressing surface-bound F1 induces high-level protection against challenge with *Y. pestis*. The available whole-cell killed vaccines are reactogenic in humans (18), and in mice they induce only partial protection against a subcutaneous challenge with 1.9×10^2 CFU of *Y. pestis* (23). The requirement for the rapid development of immunity in susceptible populations precludes the use of a killed whole-cell vaccine, which requires at least two priming immunizations over a period of 6 months and a further booster dose within the first year. Several workers have shown that antibody against the F1 antigen provides protection against parenterally administered *Y. pestis* (1, 20, 25, 32). However, even after immunization with 3 intraperitoneal doses of F1 antigen only four of five mice were protected against a subcutaneous challenge with 2×10^5 CFU of *Y. pestis* (32).

For this study, immunized mice were divided into groups of six and challenged subcutaneously at day 60 with 1.2×10^5 or 1.2×10^7 CFU of *Y. pestis* GB. The 50% lethal dose of this strain was previously reported to be approximately 1 CFU (23). All of the control mice (unimmunized or immunized with *S. typhimurium* SL3261) died by day 14 (Table 2), and *Y. pestis* was recovered when spleen samples were plated onto Congo red agar or *Yersinia* selective agar (Oxoid). Other workers have reported that mice which had been immunized with *S. typhimurium* SL3261 showed low-level protection against infection with other bacterial pathogens (11, 26), and we observed an increase in time to death compared with that for unimmunized mice after *S. typhimurium* SL3261-immunized mice were challenged with *Y. pestis*. Mice immunized with *S. typhimurium* SL3261/pAH34L survived both challenge doses, 1.2×10^5 and 1.2×10^7 CFU, without the appearance of symptoms of disease over the course of the experiment (to day 14). In contrast, mice immunized with *S. typhimurium* SL3261/pFGAL2a were only partially protected against challenge with the same doses.

The enhanced protection afforded after immunization with *S. typhimurium* expressing F1 antigen on the surface does not appear to be related to the level of expression, since the F1 antigen accumulated to a similar level when it was expressed intracellularly (22). At the time of challenge, the levels of serum antibody to F1 antigen were higher when mice had been immunized with *S. typhimurium* SL3261/pAH34L than with *S. typhimurium* SL3261/pFGAL2a, and it has been shown that the titer of antibody in serum to F1 antigen can be correlated with the degree of protection against *Y. pestis* (1). The F1 antigen has been suggested to confer antiphagocytic properties on *Y. pestis* by interfering with complement-mediated opsonization (31). It is known that the IgG2a subclass antibody is a more effective opsonin than the IgG1 subclass antibody (21), and this may explain the high level of protection seen after immunization with *Salmonella* expressing F1 antigen. This observation might also explain why F1 antigen delivered in *S. typhimurium* induces a more protective response than F1 antigen delivered in Freund's (32) or Alhydrogel (1) adjuvant, since immunization with F1 antigen induces predominantly an IgG1 response (32).

The oligomerization of *Y. pestis* F1 antigen has recently been reported (1), and the detection, by Western blotting, of multimeric forms of the F1 in crude lysates of *S. typhimurium* SL3261/pAH34L indicated that F1 antigen was assembled into oligomers. It was recently reported that antigens exported from *S. typhimurium* are more effective in inducing a protective response against listeriosis, possibly because export of the antigen more closely mimics the natural infection (15). It is possible that the surface expression of F1 antigen in *Salmonella* more closely mimics the natural presentation of F1 antigen on *Y. pestis* organisms. The oligomerization of surface-expressed F1 antigen might result in the formation of epitopes not present in the intracellular monomeric form of F1 antigen. In support of this possibility it has been reported that the F1 antigen in the killed whole-cell vaccine is less effective in inducing protective responses than purified F1 antigen, even though the whole-cell vaccines contained greater amounts of F1 antigen, a finding which was attributed to a change in the conformation of some protective epitopes in the killed whole-cell vaccines (1).

We thank K. Gilhespy, J. Trouten, P. Vesey, and J. Miller for carrying out the enzyme-linked immunosorbent assays and D. Rogers, D. Bell, and D. Whittington for technical assistance.

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Editor: J. R. McGhee