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Long-Term Immunological Memory Induced by Recombinant Oral *Salmonella* Vaccine Vectors

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We have previously shown that *Salmonella enterica* serovar Typhimurium expressing the *hagB* hemagglutinin gene from *Porphyromonas gingivalis* can induce primary and recall immune responses in serum and secretions in mice; however, the longevity of memory induced by oral *Salmonella* carriers has not been adequately demonstrated. In this study, we examined the capacity of mice to mount a recall response 52 weeks after primary immunization. Recall responses were seen in serum immunoglobulin G (IgG) and IgA following boosting at week 52, and in most cases, they were equal to or greater than the primary responses. Significant mucosal IgA recall responses in saliva and vaginal wash were also detected following boosting at week 52. In addition, there was a considerable residual response in secretions at week 51, prior to boosting. These results indicate that oral *Salmonella* vectors can induce long-term memory to recombinant HagB and are particularly effective at inducing long-lasting mucosal responses as well as at inducing the capacity for mucosal recall responses.

The mucosae serve as portals of entry for many pathogens. Because of our growing understanding of pathogenic mechanisms and host-pathogen relationships, there is increased interest in stimulating mucosal immunity as a first line of defense against colonization and establishment of disease. In order to render potential vaccine antigens immunogenic, a variety of approaches have been taken to stimulate effective mucosal immunity. These approaches include mucosal adjuvants and nonliving and live delivery systems (7, 12, 18). Avirulent *Salmonella enterica* serovar Typhimurium expressing foreign gene products has been used as a delivery system for a number of vaccine antigens (4). Live, avirulent *Salmonella* induces a diverse response including both mucosal and systemic immunity. One of the historical problems with mucosal responses to oral vaccines has been the lack of long-term mucosal memory.

The *hagB* gene codes for a hemagglutinin from the periodontopathogen *Porphyromonas gingivalis* and is a potential virulence factor (15, 19). We have previously shown that mice immunized intragastrically with *Salmonella* serovar Typhimurium expressing the *hagB* gene exhibit a vigorous serum immunoglobulin G (IgG) and IgA response to purified, recombinant HagB as well as a significant mucosal IgA response in saliva, gut secretions, and vaginal washes (5). The primary response peaks around 5 or 6 weeks after primary immunization. When mice are boosted at 14 weeks, a more rapid and intense recall response in serum and secretions is seen (16). The objectives of this study were to examine the *Salmonella* delivery system in terms of the duration of the immune response and to determine the long-term ability to mount a systemic and mucosal recall response.

Bacterial strains, plasmids, media, and culture conditions. *Salmonella* serovar Typhimurium χ4072, an SR-11 derivative (pStSR100$^-$ *gyrA1816* Δ*cya-1* Δ*crp-1* Δ*asdA1* Δ[*zhf-4*::Tn*10*]), and plasmid pYA292 (10) were provided by Roy Curtiss III (Washington University, St. Louis, Mo.). The vaccine strain χ4072/pDMD1 was constructed by electroporation with plasmid pDMD1, which expresses the *hagB* gene of *P. gingivalis*, as previously described (5). Strains were routinely grown at 37°C in Luria-Bertani (LB) medium (23). Cultures were maintained at −80°C as glycerol stocks.

Purification of HagB. Histidine-tagged HagB was purified using the QIA Express system (Qiagen Inc., Valencia, Calif.). A *Tru*9I-*Xba*I fragment of a *hagB* clone (carried on p18AX1) was subcloned into the expression vector pQE31. The recombinant plasmid was designated pQE31-TX1. Positive subclones were selected on colony blots by using absorbed antiserum to HagB (6). Cultures (500 ml) were grown with aeration at 37°C in LB broth to an A_{600} of 0.8 and then induced with 1 mM isopropyl β-D-thiogalactoside for 5 h. The cells were lysed for 1.5 h at room temperature in 6 M guanidine-HCl–0.1 M NaH_2PO_4–0.01 M Tris (pH 8.0) (buffer A). The supernatant was mixed with 8 ml of Ni-nitrilotriacetic acid resin for 1.5 h. The resin was loaded into a 1.6-cm-diameter column and washed with 10 column volumes of buffer A, followed by 5 column volumes of 8 M urea–0.1 M NaH_2PO_4–0.01 M Tris (pH 8.0) (buffer B). The column was then washed with buffer B adjusted to pH 6.3 until the A_{280} was <0.01. Attempts to refold eluted HagB by gradual dialysis were unsuccessful and resulted in precipitation of the protein. Refolding was accomplished while HagB was bound to the column. The column was equilibrated with refolding buffer (0.5 M NaCl, 10 mM Tris, 20% glycerol [pH 7.4]) containing 6 M urea. The column was then washed with a linear gradient of 6 M to 0 M urea at a flow rate of 12 ml/h over a period of 1.5 h. The histidine-tagged HagB was eluted with 250 mM imidazole and dialyzed against phosphate-buffered saline. The purified HagB appeared as a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reacted with HagB antiserum on Western blots (not shown). Routine yields were 8 to 10 mg per 500-ml culture.

Mouse immunization and sample collection. Female BALB/c, VAF/Plus mice, 6 to 8 weeks of age (Charles River, Wilmington, Mass.), were housed in the Infectious Disease Isolation Unit at the University of Florida Animal Resource Center and given food and water ad libitum. Groups of six mice were immunized with *Salmonella* serovar Typhimurium strain χ4072/pDMD1. The strain was grown as a static culture in LB broth

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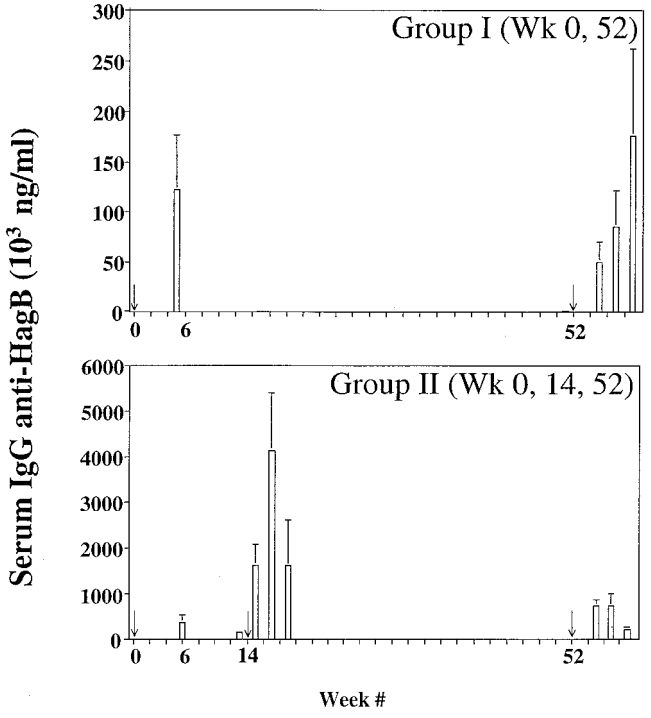

FIG. 1. Serum IgG anti-HagB levels following oral immunization and boosting (arrows) with *Salmonella* serovar Typhimurium χ4072/pDMD1. Error bars, standard errors of the means.

overnight at 37°C, diluted 1/20 in fresh LB broth, grown for ca. 4 h at 37°C to an optical density at 600 nm of 0.8, after which the culture was centrifuged and resuspended in sterile 0.1 M $NaHCO_3$ to a density of 10^{10} CFU/ml. The food supply was removed and the bedding was changed 4 h prior to immunization. Mice were immunized by gastric intubation with 10^9 cells (0.1 ml of 10^{10} cells/ml) in three doses on days 1, 3, and 5 of week 0. Boosting was carried out in the same manner.

Group I was immunized at week 0 and boosted at week 52. Week 52 was chosen to represent long-term memory since it equals approximately one-half the lifespan of a BALB/c mouse (8). Group II was immunized at week 0 and boosted at week 14 as part of a study on timing of boosting (16a) and then boosted at week 52 to assess long-term recall. Serum and saliva samples and vaginal washes were collected for evaluation of specific antibody directed against the hemagglutinin, as previously described (5, 16).

Immunoassay methods. Samples were assayed for IgG and IgA antibody to HagB on microwell plates as described previously (5) using an enzyme-linked immunosorbent assay coated with purified HagB protein. The salivary IgA anti-HagB antibody levels were normalized to amylase activity levels, and the antibody levels in vaginal washes were normalized to the total IgA to account for variable dilution encountered in secretions. The amylase activity was determined using a colorimetric enzyme assay (3).

Anti-HagB responses in serum. Mice immunized at week 0 and week 52 (Fig. 1) showed a low but measurable residual serum IgG response at week 51, just prior to boost, and a recall response at weeks 55, 57, and 59. Mice in group II, which were also boosted at week 14, showed a strong IgG recall response after the first boost and recall responses of up to ca. 1,000 ng/ml following the boost at week 52. Even though they did not exceed the peak responses seen at the earlier boost at week 14, the levels were higher than the week 6 levels and much higher than the 1-year recall levels seen in group I mice. With serum IgA (Fig. 2), antibody levels in group I mice following the boost at week 52 were higher than those for IgG. Much higher recall levels were seen in mice of group II than in mice of group I, although in this case the recall levels were comparable to those measured following the boost at week 14. Detectable levels of anti-HagB were measured at week 51 prior to boost in both groups, and they were low compared to the recall levels.

Anti-HagB responses in secretions. Recall responses in vaginal washes of single-immunized mice (Fig. 3) were comparable to primary responses, while in mice immunized both at week 0 and week 14 (group II), there were significant residual levels at week 51 as well as higher recall responses following boosting at week 52.

Evidence of the longevity of mucosal memory is most dramatically evident from salivary responses (Fig. 4). In mice of group I, residual levels were detected at week 51, prior to boosting, with subsequent recall responses which far exceeded the primary response. Likewise, in mice boosted at week 14 (group II), residual levels of IgA anti-HagB remained at week 51, and significant recall responses were seen following the boost.

Poor long-term memory with respect to secretory IgA has historically been a problem with oral immunization aimed at inducing mucosal responses. Early attempts to induce mucosal responses using orally administered, soluble proteins resulted in poor immune responses, no memory induction, and, often, oral tolerance. Various strategies including encapsulation in nonliving carriers and use of oral adjuvants have been employed to increase the immunogenicity of orally delivered antigens (12, 18).

One of the most effective oral adjuvants for the induction of

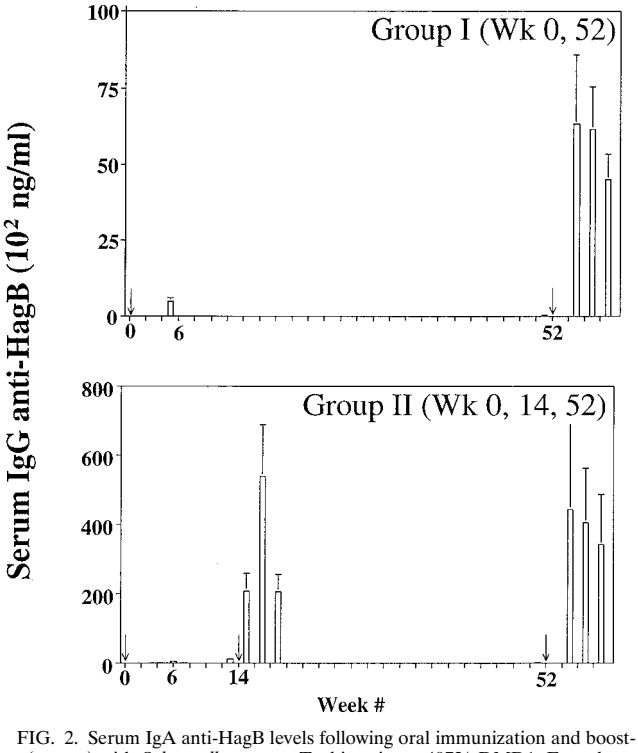

FIG. 2. Serum IgA anti-HagB levels following oral immunization and boosting (arrows) with *Salmonella* serovar Typhimurium χ4072/pDMD1. Error bars, standard errors of the means.

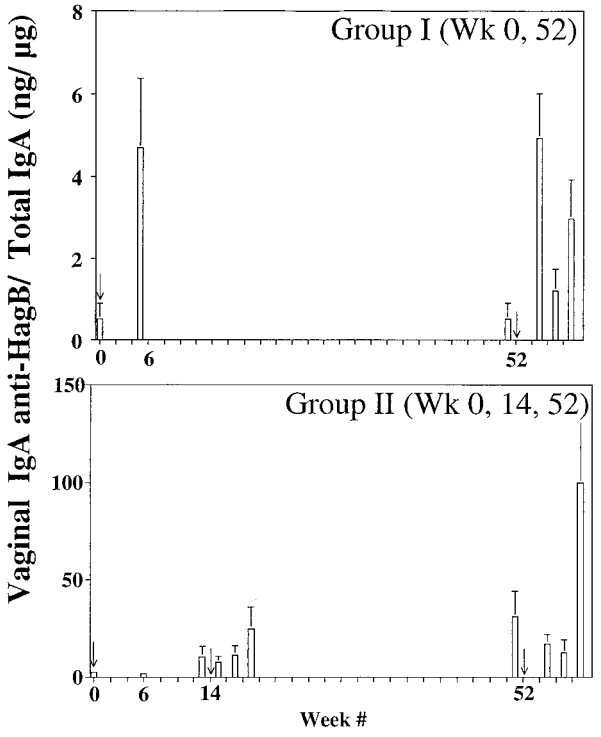

FIG. 3. Vaginal wash IgA anti-HagB levels following oral immunization and boosting (arrows) with *Salmonella* serovar Typhimurium χ4072/pDMD1. Error bars, standard errors of the means.

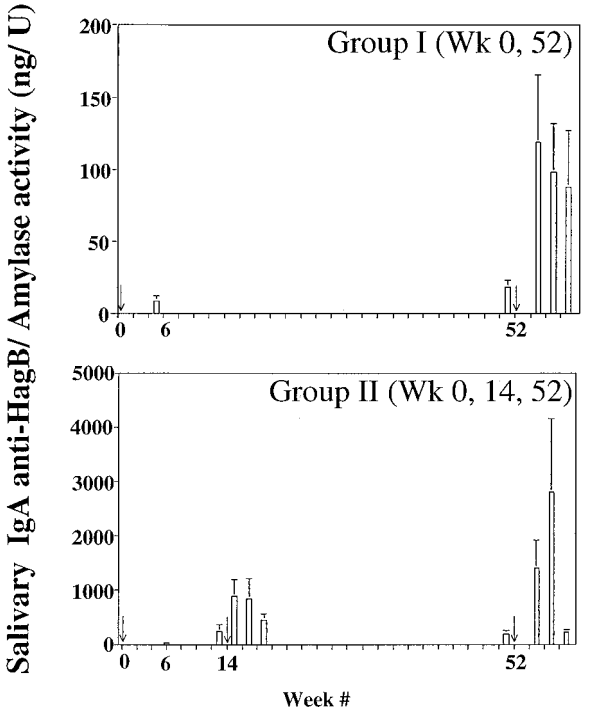

FIG. 4. Salivary IgA anti-HagB levels following oral immunization and boosting (arrows) with *Salmonella* serovar Typhimurium χ4072/pDMD1. Error bars, standard errors of the means.

mucosal responses is cholera toxin (CT) (7). Lycke and Holmgren have shown that oral immunization with CT induces memory B cells in the gut lamina propria to CT itself, which can be detected after 2 years (17). Hajishengallis et al. (11) showed that oral immunization with the saliva-binding region of *Streptococcus mutans* AgI/II genetically fused to a cholera toxin A2/B construct with CT as an adjuvant resulted in serum and salivary responses in mice which persisted to approximately 1 year; however, no recall responses were obtained following boosting at 1 year.

Fifty-two weeks correspond to nearly half the mean life span of a BALB/c mouse (8). In our experiments, while serum IgG responses after week 52 do not equal peak responses at week 14, they still reach significant levels, particularly with respect to single-immunized animals. Recall responses are seen in serum IgA, however, perhaps reflecting the mucosal inductive route. Even within the mucosal compartment, there appears to be a subcompartmentalization with respect to the optimum site of induction for various effector sites. This subcompartmentalization may be due in part to differential expression of tissue-specific adhesins on cells arising from different inductive sites (20). We have consistently detected lower levels of responses in vaginal washes than in saliva with the *Salmonella* system. This may reflect the fact that the oral route may not be optimal for inducing vaginal responses, where intranasal immunization appears to be superior (13, 14). Nevertheless, the capacity to mount a modest recall response is seen in the vaginal compartment.

What is most remarkable is the persistence and recall capacity reflected in salivary IgA responses, both in single-immunized animals and animals boosted at week 14. The *Salmonella* delivery system appears to be more capable of inducing long-term memory for salivary responses to foreign antigens, compared to the CTB subunit (11, 22, 25).

Some studies have raised concerns that repeated use of *Salmonella* vectors would not be possible due to immunity to the vector itself (1, 9, 21), while others report that prior exposure leads to enhancement of subsequent responses (2, 24). Our results from short-term recall experiments (16) and the present study support the efficacy of repeated use of *Salmonella* vectors for induction of long-term mucosal immunity.

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