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A Recombinant *Salmonella typhimurium* Vaccine Induces Local Immunity by Four Different Routes of Immunization

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Immunization of mice with an attenuated *Salmonella typhimurium* strain (Phop^c) carrying a plasmid encoding a hybrid form of the hepatitis B virus core antigen (HBc) induced specific antibody responses against the bacterial lipopolysaccharide (LPS) and HBc. Different mucosal routes of immunization, i.e., oral, nasal, rectal, and vaginal, were compared for their ability to induce a systemic as well as a mucosal response at sites proximal or distant to the site of immunization. Anti-LPS and anti-HBc immunoglobulin A (IgA) antibodies were measured in saliva, in feces, and in genital, bronchial, and intestinal secretions. Specific antibodies in serum and secretions were observed after immunization via all routes; however, the response to LPS was independent of that against HBc. In serum, saliva, and genital and bronchial secretions, high amounts of anti-HBc IgA were obtained by the nasal route of immunization. Vaginal immunization resulted in two different responses in mice: high and low. We observed a correlation between the level of specific immune response and the estrous status of these mice at the time of immunization. Rectal immunization induced high amounts of IgA against HBc and LPS in colonorectal secretions and feces but not at distant sites. These data suggest that *S. typhimurium* is able to invade different mucosal tissues and induce long-lasting local IgA responses against itself and a carried antigen after a single immunization.

To provide specific protection against the variety of pathogens which invade via the mucosa at different locations, it would be of great interest to design mucosal vaccination strategies that could induce specific secretory immunoglobulin A (sIgA) at desired sites. For instance, induction of specific sIgA in rectal and genital secretions may be important in protection against sexually transmitted pathogens such as the human immunodeficiency virus, human papillomavirus, and hepatitis B virus, while lung-specific sIgA may provide protection against respiratory infections.

Recombinant *Salmonella* strains that are attenuated yet invasive are an attractive means of delivering heterologous antigens to the mucosal and systemic immune systems (3, 5, 25). The uptake and sampling of antigens by the mucosal immune system occur at specific sites on mucosal surfaces that are identified by the presence of organized lymphoid follicles and a specialized follicle-associated epithelium containing M cells (15, 16, 21). *Salmonella typhimurium* has been suggested to preferentially cross the intestinal epithelial barrier through M cells (1, 2) before disseminating to distant sites such as the liver and spleen.

A variety of different attenuated strains of *S. typhimurium* which retain the ability to invade the gut-associated lymphoid tissue and to a lesser extent distant sites but display reduced pathogenicity even when administered at high doses have been generated (4, 14, 25). We have used a strain attenuated in its ability to survive in macrophages in vitro, CS022 [PhoP(Con) (19)], in which we introduced a plasmid (PFS14PS2) encoding a recombinant form of hepatitis B virus core antigen (HBc) (27). This protein has been shown previously to be stably expressed in *Salmonella* cells, where it assembles spontaneously into core particles. These core particles are highly immunogenic because of the presence of T helper epitopes and their highly ordered and particulate nature, which induces both T-cell-dependent and -independent antibody responses (17). HBc is efficiently taken up by antigen-presenting cells, is a weak B-cell mitogen in vitro, and induces an isotype switch (18). It has been shown that foreign B epitopes inserted within HBc gain enhanced immunogenicity (27). To date, the mucosal immune response against HBc or foreign inserted epitopes has not been investigated.

The mucosal immune response at specific sites varies with the type of antigen, the route of administration, and the species studied (9, 13, 22, 32). To date, only the oral and rectal routes of administration have been investigated with *Salmonella* vaccines. Both routes of immunization induced a systemic and mucosal response characterized by the presence of sIgA in saliva and in intestinal secretions (7, 8). Although it has been proposed that such vaccines could also induce specific sIgA in the genital tract, so far this has not been tested (6, 23).

We describe here the immune response elicited by an attenuated *S. typhimurium* strain expressing HBc administered via different routes, i.e., oral, nasal, vaginal, and rectal. We observed various levels of anti-bacterial lipopolysaccharide (LPS) and anti-HBc IgAs in all mucosal secretions analyzed, in addition to systemic IgG. We show for the first time that it is possible to (i) immunize with a *Salmonella* vaccine via the nasal and genital mucosa and (ii) induce antibodies against both the vector and the carried antigen in bronchial and genital secretions.

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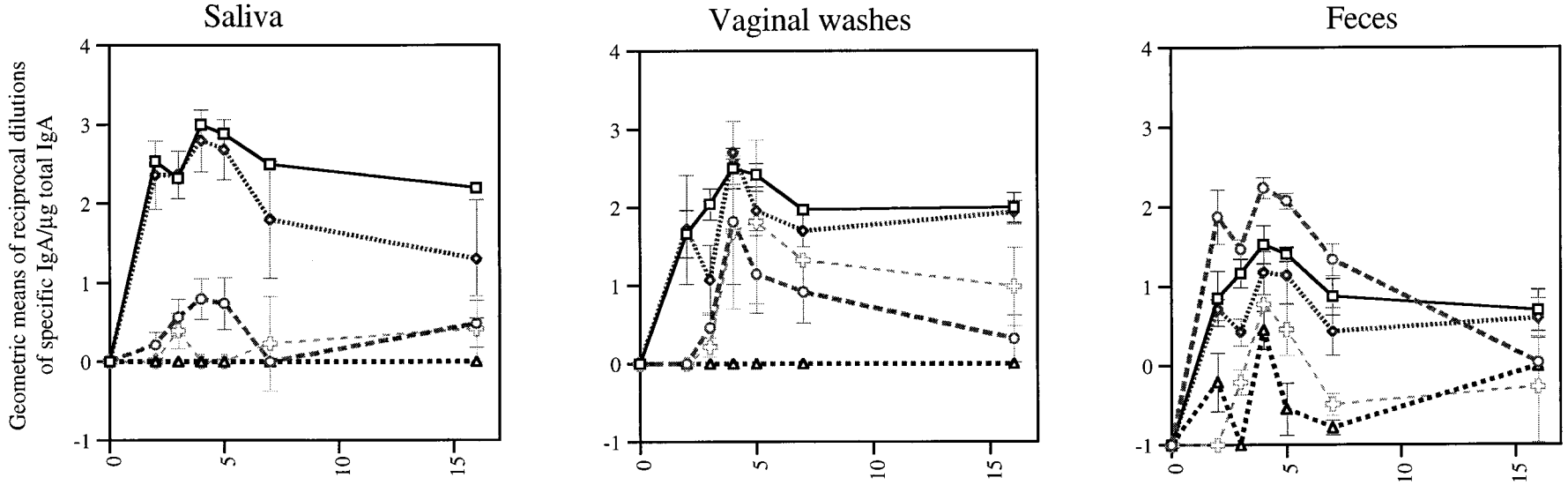
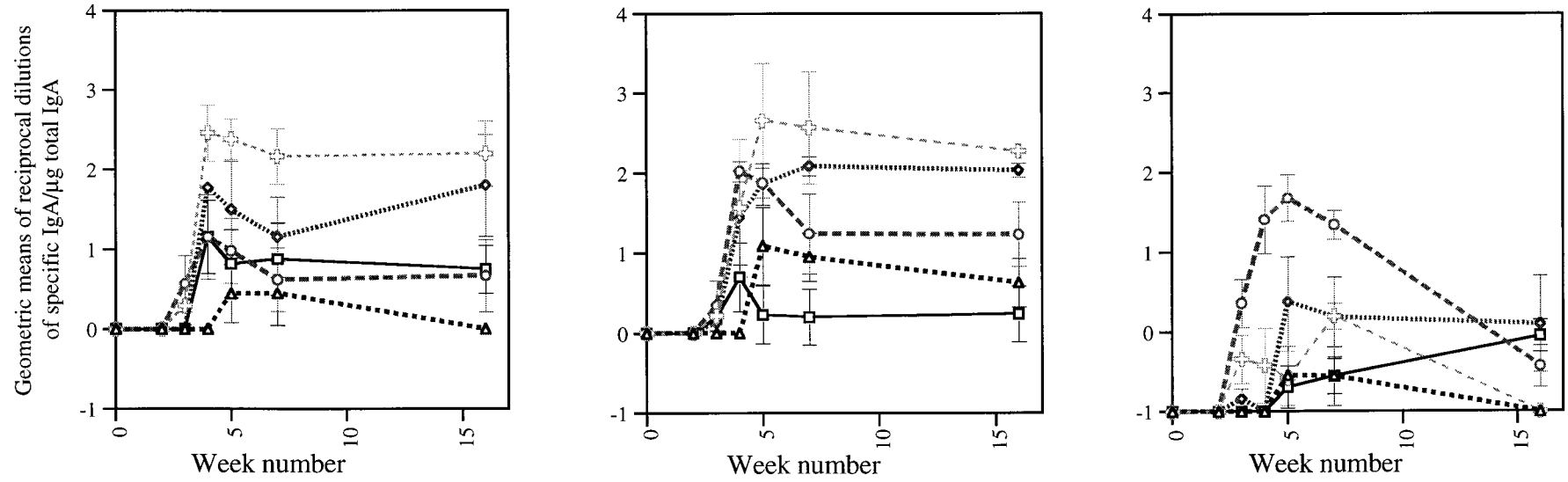
Anti-HBc IgA



Anti-LPS IgA



MATERIALS AND METHODS

Plasmid and bacterial strain used. Plasmid PFS14PS2, encoding a hybrid HBc (ayw, amino acids 1 to 155)–pre-S2 (*ayw*, 133 to 143) under *tac* promoter control (26) was electroporated as described previously (28) into bacterial strain CS022. This strain was kindly provided by John Mekalanos (Harvard Medical School, Boston); it is derived from the ATCC 14028 strain, into which the *pho-24* mutation was introduced by P22 transduction, resulting in attenuations in both virulence and survival within macrophages in vitro (19).

Immunizations. For each immunization, a single colony of Phop^c(PFS14PS2) was grown for 16 h at 37°C in 50 ml of Luria-Bertani (LB) broth with ampicillin (100 μg/ml). After 10 min of centrifugation at 6,000 × *g*, the bacterial pellet was resuspended in 1 ml of phosphate-buffered saline (PBS). The bacterial suspension contained about 5×10^{10} CFU, as estimated by plating dilutions. A 20-μl amount of the bacterial suspension was used for immunizations, with 10^9 CFU per inoculum; for lower doses, the bacterial suspension was diluted in PBS.

Six-week-old female BALB/c mice were used in all experiments. For oral immunization, mice were deprived of food 2 h before and 1 h after immunization; they were fed 30 μl of 10% sodium bicarbonate to neutralize stomach acidity 5 min prior to immunization and then given 20 μl of inoculum. For rectal immunization, mice were starved for 12 h before immunization. To perform nasal, rectal, and vaginal immunizations, mice were anesthetized by intraperitoneal injection with 100 μl (per 10 g of body weight) of both 0.2% Rompun in PBS (Bayer) and 10 mg of Ketavet (Parker Davis) per ml. Inoculum (20 μl) was gently introduced with a yellow tip into the rectum or the vagina and dropwise into one nostril. For vaginal and rectal immunizations, mice were positioned with the vagina or rectum facing upwards for about 45 min to reduce leakage of the inoculum.

Sampling of mice. Blood samples were taken from the tail veins of live animals and from the portal veins of killed animals. Salivation was induced by intraperitoneal injection of 50 μl of pilocarpine (0.5 mg/ml; Sigma) 5 min prior to sampling. Saliva samples were collected with absorbant wicks (Polytronics) as described by Haneberg et al. (9) with the following modifications. Absorbant wicks were centrifuged for 5 min at 10,000 × *g* in small 0.5-ml Eppendorf tubes pierced at the bottom with a needle and placed in large 1.5-ml Eppendorf tubes without lids. After centrifugation, the samples of saliva were collected from the bottom of the large Eppendorf tubes. Vaginal washes were obtained by gently pipetting 200 μl of PBS up and down with protease inhibitors (1:500 dilution of stock containing 5 mg of pepstatin, 5 mg of leupeptin, 5 mg of antipain, and 25 mg of benzamidine per ml in dimethyl sulfoxide; all from Sigma) with a blunt-tipped Pasteur pipette. Fecal pellets were placed in 200 μl of 5% powdered milk in PBS with protease inhibitors and dissolved by vortexing for 10 min before centrifugation at 10,000 × *g* for 10 min. The supernatant was taken and aliquoted. Mice were killed by inhalation of CO_2 4 weeks after immunization. Samples of intestinal secretions were collected as described by Haneberg et al. (9), with extraction from the wicks performed as described for saliva samples. Duodenal wicks were pooled separately from colonic wicks. Tracheal washes were performed on killed mice as described elsewhere (32). All samples were stored at −70°C.

ELISA. To determine the amount of total IgA antibodies in samples, microtiter plates were coated with 100 ng of rabbit anti-mouse Ig (Boehringer) in carbonate buffer (pH 9.6) per well overnight at 4°C. Free binding sites were blocked with 1% powdered milk and 0.1% Tween in PBS (blocking buffer) for 1 h at 37°C. Duplicate samples were diluted in blocking buffer and incubated for 2 h at 37°C. Washes were performed with PBS-Tween between incubations. The plates were incubated at 37°C for 1 h with a biotinylated rabbit anti-mouse IgA (Amersham) second antibody diluted 1:500 in blocking buffer, followed by a 30-min incubation at 37°C with a streptavidin-conjugated peroxidase (DAKO) diluted 1:5,000. Plates were developed with *o*-phenylenediamine (Sigma) and H_2O_2, and the optical density at 492 nm (OD_{492}) was measured after 15 min. A monoclonal IgA (318 μg/ml) was used as a standard, and the total amounts of IgA in samples were calculated.

For the anti-LPS enzyme-linked immunosorbent assay (ELISA), plates were coated with 100 ng (per well) of LPS coupled to methylated bovine serum albumin (BSA) (30). For the anti-HBc ELISA, plates were coated with 100 ng of recombinant HBc purified from *Escherichia coli* (28) per well. The antigen-specific ELISAs were performed as described above for IgA determination. For measurement of specific IgG, a biotinylated rabbit anti-mouse IgG (Amersham) diluted 1:500 was used as the second antibody. Endpoint dilutions of samples were carried out.

Measurement of mucosal immune response. The amounts of total IgA in mucosal secretions are highly variable. These variations reflect not only sampling methods but real variations in IgA content. The highest variation in total IgA occurred in vaginal washes, which correlates with the estrous cycle (31). The concentration of total IgA in intestinal secretions and feces was about 10 times higher than in saliva and vaginal washes (data not shown). Salivation was induced by pilocarpine, which had a diluting effect on the concentration of total IgA in saliva; however, other secretions were not affected (9). As an increase in total IgA was observed after immunization (data not shown), specific IgA values were normalized to the amount of total IgA in every secretion analyzed. The specific IgA amounts are expressed as the reciprocal of the highest dilution that yielded an OD_{492} four times that of preimmune or nonimmunized mice in the case of samples take from dead mice. These reciprocal dilutions were normalized to the amount of total IgA in each sample.

RESULTS

Expression of HBc in PhoP^c. Plasmid pFS14PS2 was transformed into the *S. typhimurium* strain PhoP^c [CS2002(PFS 14PS2)]. Stable expression of the HBc–pre-S2 fusion protein was assessed by Western immunoblotting with anti-HBc-specific rabbit serum (data not shown). This stable expression has been described previously for various other attenuated *Salmonella* strains containing the same plasmid (25, 28).

Different routes of immunization induce different patterns of mucosal immune response against HBc and LPS. Four groups of mice were immunized with the same inoculum via the nasal (four mice), rectal (five mice), vaginal (six mice) and oral (three mice) routes. We were obliged to lower the dose of the inoculum to 10^8 CFU for the nasal route, since 10^9 CFU, well tolerated by mice immunized via other routes, was lethal when administered intranasally. Samples were taken 0, 2, 3, 4, 5, 7, and 16 weeks after immunization. For each route, the average levels of anti-HBc and anti-LPS IgA elicited in saliva, vaginal washes, and feces are shown in Fig. 1.

Although the amounts of anti-HBc IgA and anti-LPS IgA in secretions cannot be compared directly, the relative IgA responses against the two antigens can be compared in relation to the different routes of immunization. For example, anti-HBc IgA elicited by nasal immunization reached similarly high amounts in saliva and vaginal washes, while low amounts of anti-LPS IgA were observed in comparison to those observed after the other routes of immunization. Rectally immunized mice had higher amounts of both anti-HBc and anti-LPS IgA in feces than mice immunized by any of the other routes tested, although the responses seemed to be short-lived. Vaginally immunized mice fell into two groups, a low- or nonresponse group (vaginal 1) and a high-response group (vaginal 2). Despite the high variability of the anti-LPS response, it appeared that the second vaginally immunized group had high anti-LPS IgA responses in saliva and vaginal washes, while anti-HBc IgAs were barely detectable in saliva. In general, the relative amounts and initial time of detection of specific IgA in secretions were different for LPS and HBc, but both responses persisted for 16 weeks except for the responses in feces of rectally immunized mice.

Different routes of mucosal immunization induce serum anti-HBc and anti-LPS IgG. The systemic response following administration of the *Salmonella* inoculum via different routes was also analyzed. The levels of anti-HBc and anti-LPS IgG in the serum of the five groups of mice is shown in Fig. 2. As already described for the mucosal response, the anti-HBc IgG response in serum differed from that against LPS when the different routes of immunization were compared. Nasal immunization appeared to be more efficient than others at inducing anti-HBc IgG in serum at week 2. At later time points, the titers of anti-HBc IgG induced by the vaginal (vaginal 2), oral,

FIG. 1. Antigen-specific IgA (anti-HBc and anti-LPS) in saliva, vaginal washes, and feces after nasal, oral, rectal, or vaginal immunization. Groups of 6-week-old BALB/c female mice were immunized with 10^8 CFU nasally (□) (four mice) or 10^9 CFU orally (◇) (three mice), rectally (○) (five mice), or vaginally (six mice). The last group was divided into two groups of three mice each: a low-responding group (vaginal 1, △) and a high-responding group (vaginal 2, ✣). Data are expressed as the geometric means of the reciprocal dilutions of specific IgA per microgram of total IgA. Error bars represent the standard errors of the means.

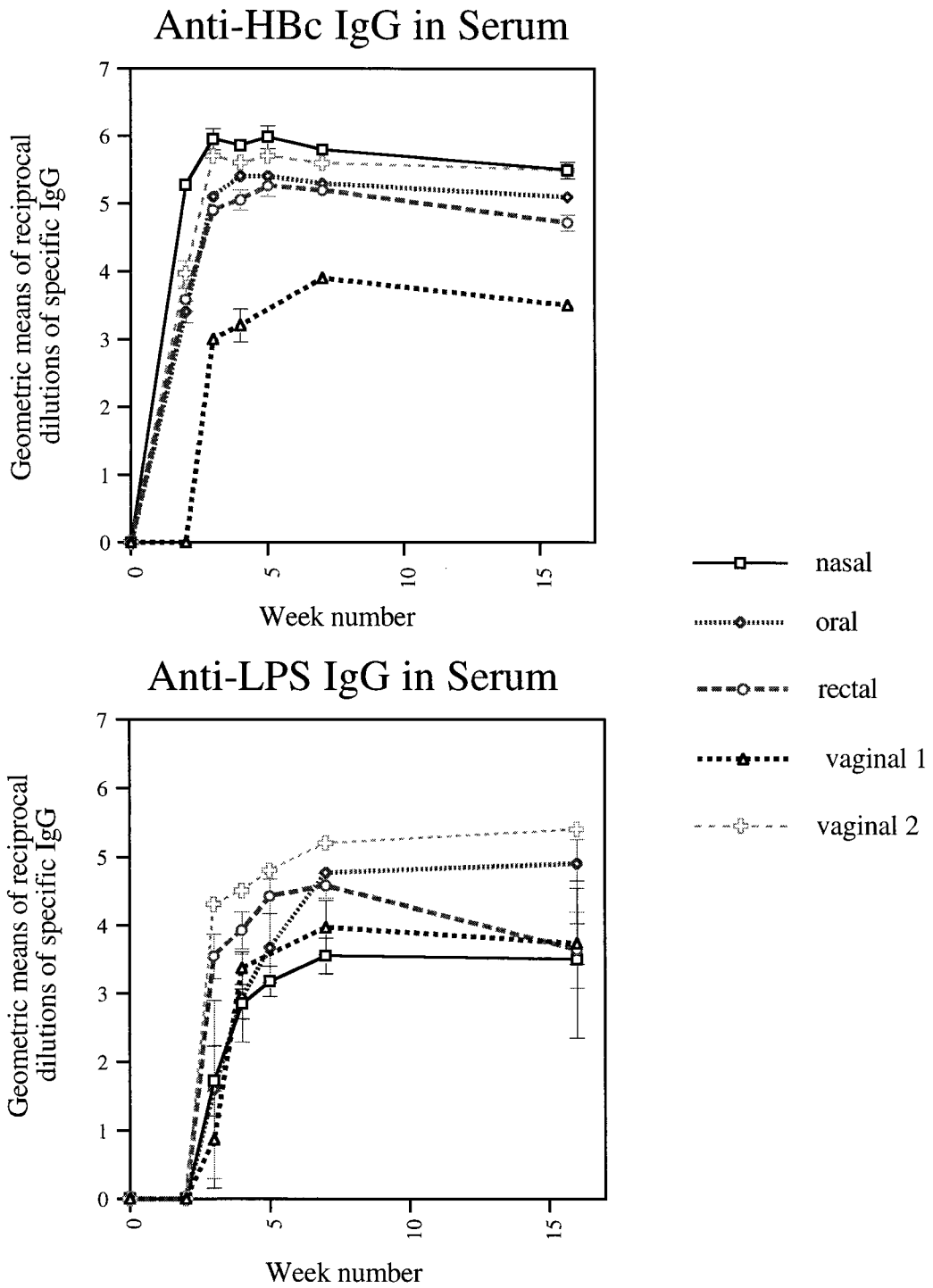


FIG. 2. Antigen-specific IgG (anti-HBc and anti-LPS) in serum after nasal, oral, rectal, or vaginal immunization. Groups of 6-week-old BALB/c female mice were immunized with 10^8 CFU nasally (four mice) or 10^9 CFU orally (three mice), rectally (five mice), and vaginally (six mice). The last group was divided into two groups of three mice each: a low-responding group (vaginal 1) and a high-responding group (vaginal 2). Data are expressed as the geometric means of the reciprocal dilutions. Error bars represent the standard errors of the means.

and rectal routes reached levels similar to those obtained by the nasally immunized mice. The anti-HBc IgG response in vaginally immunized mice (vaginal 1) was 100- to 1,000-fold lower than that elicited by the other groups.

The anti-LPS IgG response was highly variable in all groups of mice except the second group of vaginally immunized mice, which had a consistently high response. All routes of immunization induced a long-lasting (at least 16 weeks) IgG response against both HBc and LPS.

Oral and nasal immunization with different doses of inoculum. We observed that the lethal dose of the attenuated strain Phop^c given nasally was lower than that by the other routes

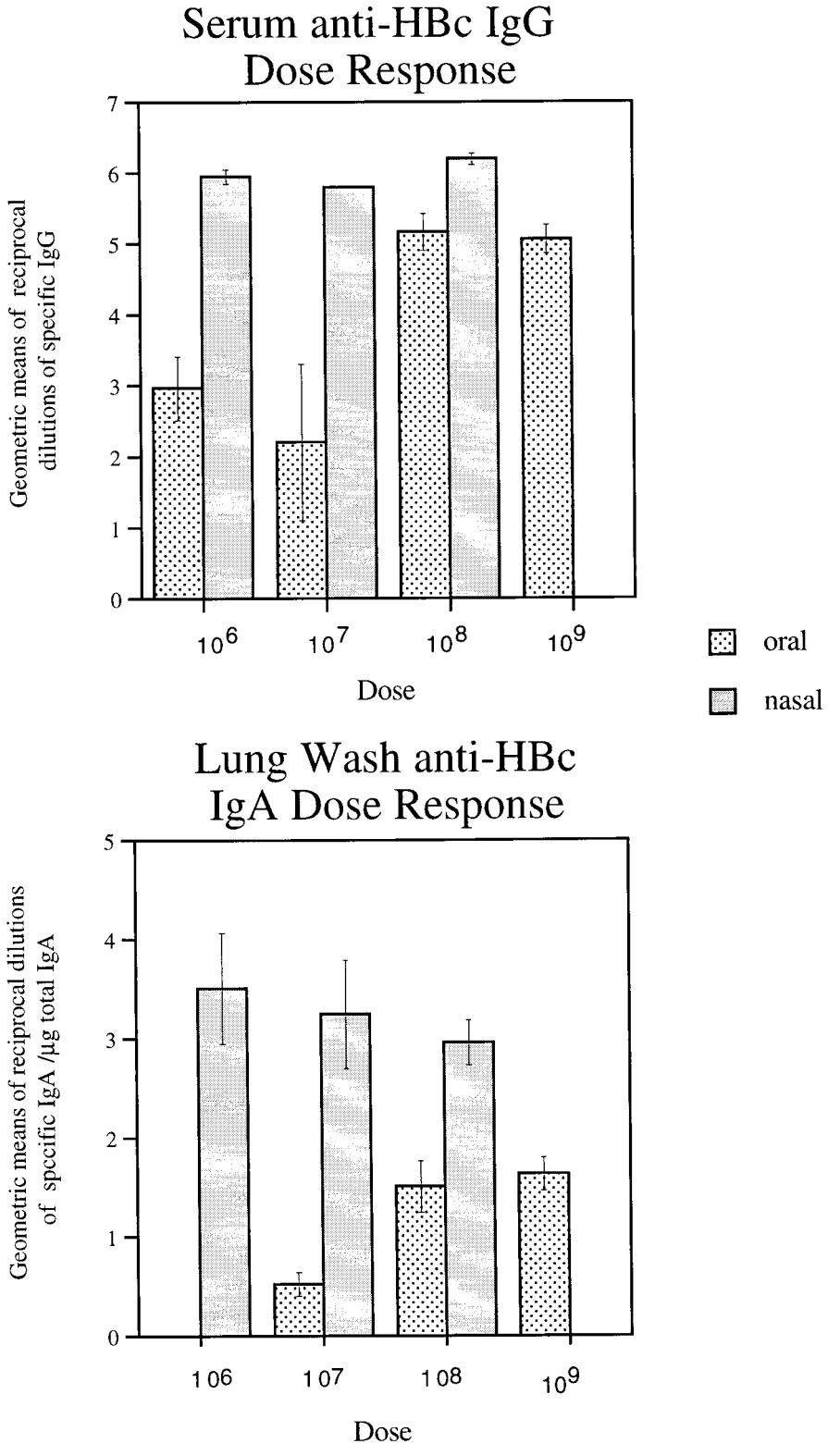


FIG. 3. Anti-HBc immune responses in serum and lung washes after oral or nasal administration of different doses of inoculum. Six-week-old BALB/c female mice were immunized in groups of three with different doses of inoculum nasally or orally. Data are expressed as the geometric means of reciprocal dilutions of specific IgG for serum and as the geometric means of reciprocal dilutions of specific IgA per microgram of total IgA for lung washes. Error bars represent the standard errors of the means.

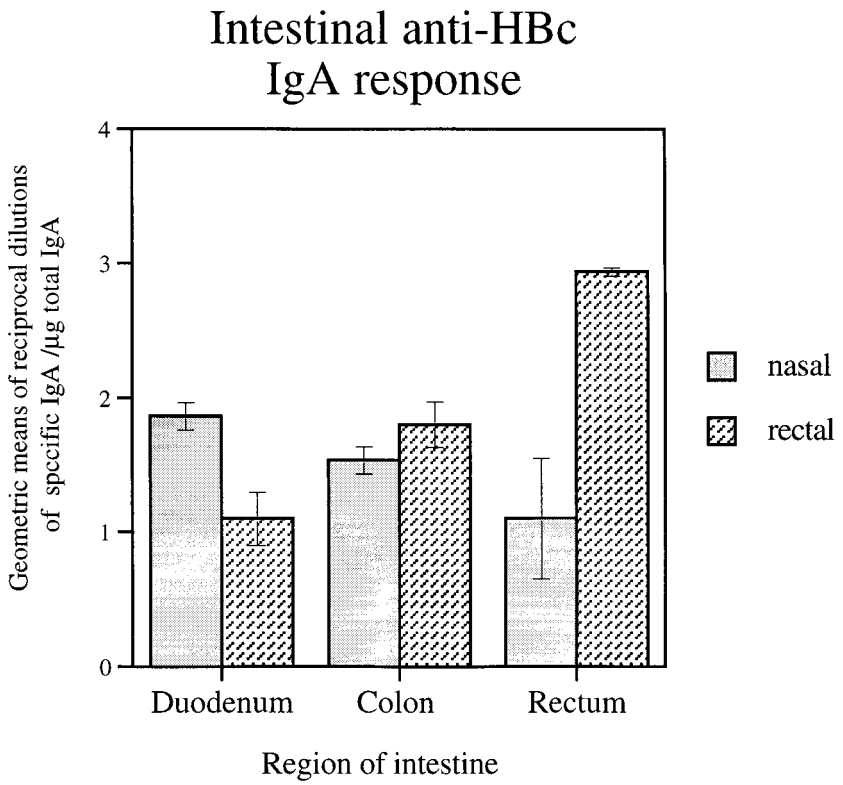


FIG. 4. Anti-HBc IgA responses along the intestine after nasal or rectal immunization. Six-week-old BALB/c female mice were immunized in groups of three with 10^8 CFU nasally or 10^9 CFU rectally. Data are expressed as the geometric means of the reciprocal dilutions of specific IgA per microgram of total IgA. Error bars represent the standard errors of the means.

tested. A dose of 10^9 CFU consistently killed the animals and obliged us to use a lower dose for this route. In order to validate our comparison of the different routes of immunization, we compared the systemic responses against HBc in orally and nasally immunized mice with the same inoculum at several different doses. The average response at week 4 is shown in Fig. 3. Interestingly, at all doses tested via the nasal route, the titers of anti-HBc IgG in serum were similarly high with low variability, while oral doses of 10^9 and 10^8 CFU induced high responses which, despite high variability, decreased abruptly when 10^7 or 10^6 CFU was administered. We noticed the same phenomenon when we analyzed the anti-HBc IgA responses, as illustrated by lung washes of these mice (see below and Fig. 3).

Lung immune response induced by oral or nasal immunization. Both oral and nasal immunizations induced high IgA responses against HBc in saliva. It has been suggested that the saliva IgA response correlates with that of the lung, constituting an upper-airway response. We therefore analyzed anti-HBc IgA in lung washes of orally and nasally immunized mice (Fig. 3). At a dose of 10^8 CFU, nasal immunization induced a high anti-HBc IgA response in lung washes, similar to that measured in saliva, while oral immunization induced a much lower anti-HBc IgA response in lung washes.

Intestinal immune response induced by nasal or rectal immunization. Rectal immunization induced the highest amount of specific antibodies in feces, as shown in Fig. 1. To determine the origin of the secretions that contributed to the high amount of IgA found in feces, we sampled separate parts of the intestinal tract after rectal or nasal immunization. Three mice were immunized rectally with 10^9 CFU, and three mice were immunized nasally with 10^8 CFU. The animals were killed at week 4, and the intestinal secretions along the length of the gut were sampled by the wick method (9). Duodenal, colonic, and rectal secretions were analyzed separately. The average levels of anti-HBc IgA are shown in Fig. 4. The rectal route of immunization induced higher amounts of anti-HBc in the rectal secretions than did nasal immunization, reflecting the higher amounts of specific IgA found in feces. Anti-HBc IgA levels in duodenal and colonic secretions were similar in mice immunized by either route.

DISCUSSION

To determine better vaccine strategies for inducing immune responses at defined locations, the immune responses against both the bacterial vector and a carried antigen were analyzed. We showed that by using appropriate routes of administration, the vaccine was successful at inducing mucosal immune responses in all secretions studied. A discrepancy in the immune responses against LPS and HBc was found in relation to the route of administration, with the exception of the IgA response in feces. For example, while nasal immunization induced higher systemic and mucosal responses against HBc than other routes of immunization, it induced lower responses against LPS. In contrast, vaginal immunization induced higher systemic and mucosal responses against LPS and lower mucosal responses against HBc. In addition, in all cases the systemic response against LPS was more variable than that against HBc. These findings may reflect separate mechanisms underlying the immune responses against the two antigens after induction at different sites. The two antigens are different in both structure

and location within the bacterium: HBcAg is a recombinant protein expressed in the bacterial cytosol and is both a T-cell-dependent and -independent antigen (17), while LPS is a lipopolysaccharide antigen present at the bacterial cell surface and is a T-cell-independent antigen (20). These different features of the two antigens could contribute to the different antibody responses observed against them. The immune response to both a carried antigen and the carrier itself is also related to the ability of the carrier strain to invade, replicate, and persist in host tissues, in addition to the stability and the level of expression of the carried antigen achieved. Any of these features could vary with the site of immunization used.

Salmonella organisms normally invade via the oral route, where they cross the intestinal epithelial barrier preferentially through M cells (1, 2). Sampling of antigens by M cells results in the priming of antigen-specific B cells in the associated lymphoid aggregates (10). These cells subsequently migrate from the site of induction and mature into plasma cells that produce IgA. The migrating IgA-expressing B cells home preferentially to sites close to that of induction but also to distant mucosal sites (15). Such lymphoid-inductive sites (with M cells) have been described for the digestive tract (gut-associated lymphoid tissue), the bronchial tract (bronchus-associated lymphoid tissue [29]), and the nasal duct (nasal-associated lymphoid tissue [11]). Indeed, we have shown that a *Salmonella* vaccine was successful at eliciting a mucosal immune response after immunization at oral, nasal, and rectal sites. In addition, salmonellae also elicited an immune response after vaginal immunization, in spite of the fact that the genital mucosa is devoid of M cells and classical sampling sites. Parr and Parr (24) have described lymphoid nodules in the adventitia lateral to the urethra and in addition have shown that the iliac, renal, and caudal lymph nodes drain the vagina. However, it is not known whether the former can act as inductive sites or whether specific IgA responses are mounted at the latter distant-draining lymph nodes.

Vaginal immunizations with salmonellae were only successful at inducing an immune response when the mice were at late metestrus or diestrus (vaginal 2; Fig. 1). This also occurred when the two uterine horns were ligated to prevent the salmonellae from directly reaching the peritoneal cavity (data not shown). Parr and Parr (24) have also shown that tracers are taken up preferentially at diestrus by Langerhans cells in the vagina. Interestingly, Young et al. (33) have shown that there is a relationship between the estrous cycle of the mouse and the thickness of the epithelium in addition to the number of Langerhans cells present within it. At late metestrus and early diestrus, when the salmonellae were able to induce an immune response, the epithelium is relatively thin and the number of Langerhans cells is high. Whether these observations are correlated with *Salmonella* invasion is currently under investigation.

If we consider the overall mucosal immune responses at different locations, with the exception of the genital response, we can conclude that the oral, nasal, and rectal routes of immunization induced higher immune responses at sites proximal to the site of induction than at distal sites. Indeed, nasal immunization induced high responses in saliva and lung washes, oral immunization induced them in saliva, and rectal immunization induced them in colonorectal secretions. In contrast, the genital inductive and effector sites are different, i.e., oral and nasal immunization induced specific IgA in high amounts in genital secretions, whereas vaginal immunization induces high anti-LPS responses in saliva.

Our studies demonstrated that, overall, the nasal route of immunization was the most efficient at inducing systemic and mucosal immune responses to the carried antigen, even when the dose was reduced to 10^6 CFU. It is not clear which inductive site(s) is responsible for mounting an immune response after nasal immunization. For example, whether an inoculum can reach the lung after nasal immunization is questionable (12, 32). Therefore, we inoculated mice nasally with Chicago blue dye (Sigma) 5 min prior to killing and dissecting them. The dye reached both the lung cavity and the intestine, suggesting that salmonellae are both inhaled and swallowed and therefore could contact the nasal-, bronchus-, and gut-associated lymphoid tissues after nasal immunization (data not shown). In addition, preliminary results suggest that the bacteria colonize the lungs and persist there for at least 3 weeks after nasal immunization (data not shown). These findings suggest that nasal immunization with salmonellae probably results in induction of nasal-, bronchus-, and gut-associated lymphoid tissue.

In summary, when comparing the different routes of immunization, oral and nasal administration are similar in their ability to induce high amounts of anti-HBc IgA in saliva and genital secretions. Rectal immunization induces high amounts of specific IgAs in rectal secretions, and nasal immunization induces high amounts in lung washes. In conclusion, by using recombinant *Salmonella* vaccines to protect against pathogens which invade via the respiratory or genital tract, nasal immunization may be more efficient, whereas to protect the rectal mucosa, rectal immunization may be required.

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