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Oral Vaccination of Calves with an Aromatic-Dependent *Salmonella dublin* (O9,12) Hybrid Expressing O4,12 Protects against *S. dublin* (O9,12) but Not against *Salmonella typhimurium* (O4,5,12)

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Three groups of six calves each, 5 to 7 weeks old, were orally vaccinated with the live aromatic-dependent ΔaroA *Salmonella dublin* (O9,12) hybrid strain SL7103 with the O4,12-specifying *rfb* gene cluster from *Salmonella typhimurium*. SL7103 was given in three weekly doses, increasing from 2 × 10⁶ to 1 × 10¹⁰ bacteria per ml, was well tolerated, and caused mild, short-term temperature increases which diminished with each immunization. The strain was shed for up to 1 week. Strain SL7103 elicited significant (P < 0.001) and equal anti-*S. dublin* and *S. typhimurium* lipopolysaccharide serum antibody responses and skin delayed-type hypersensitivity immune responses. Six vaccinated calves orally challenged with 10¹⁰ CFU (equivalent to 1,000 50% lethal doses) of the virulent parent strain *S. dublin* SVA47 were protected and experienced only transient fever and mild mucoid diarrhea. However, six vaccinated calves orally challenged with 3 × 10⁹ CFU and another six challenged with 3 × 10⁸ CFU (equivalent to 1,000 50% lethal doses) of the virulent *S. typhimurium* SVA44 became bacteremic with a profuse hemorrhagic diarrhea and had to be sacrificed within 2 to 7 days. The results suggest that the *S. typhimurium* antilipopolysaccharide immunity was insufficient to provide a solid protective efficacy against oral *S. typhimurium* infection. The immunohistopathological examination revealed that *S. typhimurium* SVA44 could be found in all layers of the intestinal mucosa and the lymphatic tissues of the Peyer’s patches. In contrast, *S. dublin* SVA47 was found predominantly in the columnar enterocytes of the jejunum and ileum and the follicle-associated epithelium over the Peyer’s patches. In addition, SVA47 was found in the glandular tissues of the duodenal and tonsillar areas and in the lungs. This suggests that the *S. typhimurium* and *S. dublin* strains have different virulence traits determining their tissue localization and dissemination.

Salmonella infections in cattle are still a worldwide problem, and the bacteria have established themselves as enzootic organisms in several regions. Two species (or serovars), *Salmonella typhimurium* and *Salmonella dublin*, account for the majority of cases (6).

*S. typhimurium* differs from *S. dublin* in its O-antigenic makeup: *S. typhimurium* belongs to serogroup BO defined by the O4 antigen, and *S. dublin* belongs to serogroup DO with the O9 antigen defined as serogroup specific (11). The structures of the O-antigenic polysaccharide chain of both serogroups are known, and they share the α-D-mannose-1→4α-L-rhamnose-1→3α-D-galactose trisaccharide of the repeating unit and differ only in the nature of the 3,6-dideoxyhexosyl α-L,3-linked to the D-mannose residue: it is abequose in serogroup BO and tyvelose in serogroup DO (15). The tetrasaccharide repeating units are joined with α1,2 linkages between the D-galactose and D-mannose residues to form the polymerized O-antigenic polysaccharide chains.

The O-antigenic polysaccharide chain is part of the cell envelope lipopolysaccharide (LPS) of enterobacteria. Immunity directed against the LPS is thought to be of major importance in the host defense against salmonellae (12, 18). Safe live oral vaccines against *S. typhimurium* and *S. dublin* have been constructed and shown to confer protection against experimental infection with virulent wild-type strains (2, 3, 5, 7, 22–24, 26). In most instances, the host defense has been shown to be more or less strain specific so that *S. typhimurium*-immunized calves were protected against *S. typhimurium* but not *S. dublin* and vice versa. However, in one study, parenteral vaccination with an aromatic-dependent *S. dublin* was reported to protect against oral challenge with either virulent *S. dublin* or *S. typhimurium* (23).

We were recently successful in constructing partial diploid strains which, through introduction of the genes specifying the synthesis of the O-antigenic repeating unit (*rfb*) of serogroup D into serogroup B strains, simultaneously expressed both the O4 and O9 specificities in almost equal amounts (9, 25). One construct described in the preceding communication (16), *S. dublin* (O9,12) SL5631 with the *rfb* cluster of *S. typhimurium* (O4,5,12), labelled SL7103, was found to elicit a protective immunity in NMRI mice immunized and challenged intraperitoneal with either *S. dublin* or *S. typhimurium*.

In this communication we describe the use of the hybrid SL7103 strain as an oral live vaccine in 5- to 8-week-old calves. The well-being of the calves as well as their rectal temperatures, the excretion of SL7103, and the immune response against the LPS antigens was monitored. Vaccinated calves were also challenged with a virulent wild-type strain, either *S. dublin* SVA47 or *S. typhimurium* SVA44.

MATERIALS AND METHODS

Experimental animals. Swedish Red and White breed calves (male and female), 5 to 8 weeks old and weighing 40 to 60 kg, were used. The calves came from salmonella-free...
herds with no previous history of salmonellosis. All calves were also shown to be free of salmonella in three fecal cultures and showed negative results in humoral and cell-mediated immunity tests.

The vaccination and challenge experiments were done in an infectious unit on three groups of calves with six animals in each group. The calves were kept separated from each other in single boxes. All calves had water, hay, and concentrated feed ad lib. In addition, they were given 2.5 liters of a milk replacer (Kalv 1; Lantmännen, Uppsala, Sweden) twice daily.

**Bacterial strains.** The hybrid vaccine strain, *S. dublin* SL7103, is described in the accompanying article (16). The *aroA* S. *dublin* strain SL5631 was available from a previous study (21).

The virulent challenge strains *S. dublin* SVA47 and *S. typhimurium* SVA44 belong to the strain collection of the National Veterinary Institute, Uppsala, Sweden. The antigenic formula of SVA47 is O:1,9,12;H:gp,–. The strain possesses a 50-MDa plasmid and is resistant to sulfonamides and mecillinam. The SVA44 strain is O:4,5,12:H;I:1,2 phase type 8 (14), H2S positive, and grows on Simmons citrate agar at 37°C. SVA44 is resistant to streptomycin and sulfamethoxazole. For a comparison of the growth characteristics, the parent strain *S. dublin* SVA47, its aromatic-dependent mutant *S. dublin* SL5631, and the aromatic-dependent hybrid *recaI*-negative mutant SL7103 were grown overnight in brain heart infusion (BHI) broth (Difco B37). The overnight culture was diluted to an *A*950 of 0.01 in BHI broth and incubated in flasks without aeration at 37°C. At intervals, the optical density at *A*950 was determined and the samples were diluted in phosphate-buffered saline (PBS; pH 7.4) for determination of the viable count on nutrient agar (Difco).

For the challenge infection, the strains were grown in BHI broth (Difco B37) in an unshaken culture for 18 h at 37°C. The challenge doses were estimated by the *A*950 and verified by agar plate count. Each calf was given the challenge dose orally in approximately 2.5 liters of milk replacer (Kalv 1).

**Clinical examination.** All calves were examined twice daily starting 1 week before experimentation and throughout the study period, and all clinical reactions including the rectal temperature were recorded.

**Bacteriological examinations.** Rectal fecal samples were taken daily for bacteriological examination. The qualitative analysis included enrichment in selenite broth (Difco B37) with subculturing on brilliant green agar (Oxoid CM329). For quantitative analysis, a weighed portion (0.5 to 1.0 g) was suspended in PBS and diluted in PBS in 10-fold steps, and 0.1 ml of each dilution (undiluted to 10−3) was inoculated onto brilliant green agar plates and incubated for 18 h at 37°C. Suspected salmonella colonies were enumerated and serologically verified (by Kauffmann-White classification). Blood samples (10 ml) were inoculated into blood culture flasks with a broth as well as an agar phase (National Bacteriological Laboratory, Stockholm, Sweden) at 37°C for 48 h. Subcultures were done on brilliant green agar plates and qualitatively examined for the presence of salmonellae as described above.

**Pathological examination.** Immediately after euthanasia (bulb pistol, bleeding), all calves were autopsied. The organs were removed and tissue specimens were taken in the following order: liver, spleen, duodenum, jejunum, ileum, colon (within 5 min). Each specimen not bigger than 2 cm× and 5 mm thick was immediately placed in a separate jar with 100 ml of 10% buffered formalin to ensure rapid, even fixation. Sections were stained with hematoxylin and eosin for morphological examination and by the peroxidase-antiperoxidase (PAP) technique for immunohistological detection of *Salmonella* species as previously described (1). Two sections, 4 to 6 μm thick, of each tissue specimen were cut, deparaffinized with xylol, and rehydrated through graded alcohols to water. Endogenous peroxidase activity was quenched with a solution of 1.5 ml of 30% hydrogen peroxide in 48.5 ml of Tris-buffered saline (pH 7.6) for 20 min. To prevent the primary antibody from being unspecifically adsorbed to charged sites, the sections were incubated with a dilution of bovine serum albumin for 15 min. Excess liquid was tapped off, and one of the two sections of each specimen was incubated for 45 min with a primary mouse antisalmonella (O:9, O:4, O:6,7) monoclonal antibody. The other section was used as a negative control and included in all steps except the incubation with the primary antibody.

After the nonbound mouse antisalmonella antibodies were rinsed off, another incubation for 15 min with normal non-immune serum was performed, excess liquid was removed, and an incubation for 30 min with the link antibody, a rabbit anti-mouse immunoglobulin G (IgG) (Dakopatts, Hågersten, Sweden), diluted 1:20, was carried out. After being rinsed, the sections were finally incubated for 30 min with mouse PAP complex serum (Dakopatts) diluted 1:100. A colored end product was obtained by placing the slides for 8 min in a solution consisting of 24 mg of diaminobenzidine-tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) dissolved in 40 ml of Tris-buffered saline (pH 7.6), with 40 μl of 30% hydrogen peroxide added just before use. Finally, the sections were rinsed in water, counterstained with Mayer’s hematoxylin, and coverslipped. All incubations were carried out at room temperature (22°C), and between the steps the sections were rinsed with Tris-buffered saline. Positive controls were included in every staining batch. Rabbit polyclonal O:4- and O:9-specific antisera elicited against synthetic glycoconjugates and mouse monoclonal antibodies also specific for O4 and O9 epitopes were available from previous investigations (20, 25).

Specimens for qualitative and quantitative bacteriological examination were taken with disposable plastic spoons from each of nine lumen surface locations along the alimentary tract (tonsil, gallbladder lumen, duodenum, jejunal, ileal, caecal, and colon lymph nodes; lung; thymus; liver; gallbladder; spleen; rumen; abomasum; duodenum; jejunum; ileum; cecum; and colon). The tissue specimens were weighed (samples up to 25 g were taken), homogenized in PBS with a pestle and mortar, and diluted in PBS. The dilutions were cultured as described above.

**Immunological investigations.** The enzyme immunonassay was performed as described earlier (10). The *S. typhimurium* SH4809 (O4,5,12), *S. enteritidis* SH1262 (O9,12), and *Salmonella thompson* IS40 hot-phenoil-water-extracted LPS antigens were available from previous investigations (19). The class-specific immunoglobulin titers were estimated by using mouse monoclonal antibodies specific for the heavy chains of the bovine immunoglobulins, with subsequent detection by using a goat anti-mouse immunoglobulin-alkaline phosphatase conjugate as described earlier (20). All enzyme immunonassay titers are expressed as relative titers, i.e., the absorbance value of the dilution multiplied by the dilution factor (1,000).

**Skin tests.** A crude cell homogenate was prepared from
each of the S. dublin SVA47 and S. typhimurium SVA44 strains as described previously (19). The homogenate, which contained a mixture of outer membrane proteins, LPS, and cytoplasmic materials, was used in a concentration of ca. 200 μg (dry weight) per ml and contained 60 μg of protein per ml. The skin of the upper lateral part of the neck of each calf was depilated, and the injection site was marked. The double skinfold thickness was measured by using a slide caliper before and 48 h after an intradermal injection of 0.3 ml of the SVA47 or SVA44 cell homogenate. An increase of 4 mm or more in double skinfold thickness was considered a positive reaction.

Statistical analyses. Student’s t test was used for the statistical analyses (4).

RESULTS

Growth of S. dublin SVA44 and auxotrophic vaccine strains SL5631 and SL7103. The growth characteristics of the wild-type parent S. dublin SVA47 in BHI broth in submerged culture without oxygenation at 37°C was compared with those of its aromatic-dependent aroA mutant SL5631 and the hybrid O4-O9 strain SL7103, which is ΔaroA and carries the recA1 mutation. Strains SVA44 and SL5631 grew at the same rates and reached optical density plateau values after approximately 5 h (Fig. 1) and bacterial counts of ca. 2 × 10⁹ to 4 × 10⁹ CFU/ml. Strain SL7103 grew at the same rate, but its optical density plateau value was ca. 0.2 unit lower than that of SVA47 and SL5631 (Fig. 1) and its bacterial count leveled off at ca. 5 × 10⁶ CFU/ml.

Immunization with the hybrid strain S. dublin SL7103. A total of 18 calves were given the hybrid vaccine strain SL7103 orally in three separate experiments. The vaccine was given in three doses on days 0, 7, and 14 (ca. 2 × 10⁹ bacteria on day 0, ca. 1 × 10¹⁰ bacteria on day 7, and ca. 1 × 10¹¹ bacteria on day 14) (Fig. 2). The calves tolerated the vaccine, continued to feed well, and gained weight. Only slight increases of the rectal temperature, with maxima on days 2 (mean, 0.5°C; standard deviation, 0.7) and 9 (mean, 0.2°C; standard deviation, 0.1), lasting about 4 days were recorded (0.01 < P < 0.1). After the third vaccination with the highest dose on day 14, no temperature increases were observed.

Six of the 18 calves showed mild and transient diarrhea during the immunization period. The diarrhea was intermittent and lasted only 2 to 3 days: it was seen in three calves on days 2 and 4 and in five calves on days 9 and 10. No diarrhea was observed after the third vaccination. The excretion of SL7103, recovered by quantitative culture of fecal specimens, showed peak values the day after the first, second, and third immunizations of 6 × 10⁵, 4 × 10⁶, and 7 × 10⁶ CFU/g, respectively (Fig. 2). Thus, there was no increase in the mean number of bacteria excreted in spite of a 10-fold-higher dose at each successive vaccination. The number of excreted SL7103 organisms fell rapidly, and the mean numbers were less than 100 CFU/g within 3, 4, and 3 days after the first, second, and third oral doses, respectively. However, a variation from 0 to 10⁸ CFU/g was recorded on days 3 to 7, 11 to 14, and 16 to 18. From day 20, the vaccine strain was recovered only after enrichment. Only one calf was found shedding the vaccine strain at the time of challenge (day 28).

The SL7103 vaccine strain was recovered in blood samples from three calves on day 1 and from one each on days 10 and 17 (five separate calves with a single episode each). None of the calves showed any clinical signs of bacteremia.

Challenge with virulent S. dublin SVA47. The first six calves were infected with a dose of 10¹⁰ bacteria of the virulent S. dublin SVA47 strain which corresponds to approximately 1,000 50% lethal doses (LD₅₀S) (Fig. 3A). None of the calves became ill, but a marked transient increase of the rectal temperature, with a maximum mean value of 40.6°C (standard deviation, 0.8) on day 2 after the challenge, was recorded. The increases were significant (0.001 < P < 0.01) on days 2 and 3 after challenge. The temperature had returned to the normal level on day 5. The day after the infection, all calves began to excrete semiliquid mucoid stools which never became mixed with fibrin or blood. The loose stools lasted for only 1 day in four calves and for 2 and 4 days in the other two. The number of excreted SVA47 decreased rapidly. At 6 days after the challenge infection, the mean value had already dropped from the top value of 2 × 10⁶ CFU/g of feces on day 1 to less than 1 × 10⁵ CFU/g of feces. No salmonellae were recovered in the feces from any calf at the time of sacrifice 21 days after the challenge infection (Fig. 3A).

The challenge strain was not recovered in any of the blood cultures taken on each of the first 3 days after challenge. The gross pathological findings at the time of sacrifice of the clinically healthy calves 3 weeks after challenge were
limited to moderately enlarged spleens and mesenteric lymph nodes. In the histopathological examinations, lymphatic hyperplasia in the spleen, tonsils, intestinal lymphatic tissues, and mesenteric lymph nodes was seen (not shown). Slightly shortened villi, an increased number of migrating leukocytes, and small intraepithelial accumulations of degenerated neutrophils and enterocytes were also found in the mucosa of the terminal small intestine.

The bacteriological examination of specimens taken from the 29 different specimens from every calf at necropsy showed growth of the S. dublin SVA47 challenge strain in the liver specimen from one calf only.

**Challenge with virulent S. typhimurium SVA44.** Two groups of the vaccinated calves were infected with the virulent S. typhimurium SVA44. A group of six calves was given ca. $3 \times 10^9$ SVA44 bacteria, corresponding to ca. 10,000 LD$_{50}$s (19). All calves developed severe hemorrhagic diarrhea and had to be sacrificed within 48 h after challenge. A second vaccinated group of six calves was subsequently given ca. $3 \times 10^8$ S. typhimurium SVA44 bacteria, corresponding to approximately 1,000 LD$_{50}$s (Fig. 3B). All calves became severely affected by an enteritis which initially was catarrhal but changed to hemorrhagic within 3 days. All six calves had to be sacrificed within 3 to 7 days. At day 1 after challenge, the calves already had significantly increased ($0.001 < P < 0.01$) rectal temperatures. The mean fecal numbers reached a maximum on day 3 of $8 \times 10^8$ CFU/g (Fig. 3B). SVA44 was recovered from the blood of five of the six calves.

**FIG. 2.** Fecal excretion of S. dublin SL7103 after oral ingestion of three vaccine doses in 18 calves. The fecal counts are expressed as the mean number of SL5631 bacteria per gram of feces (the vertical bars show the maximum numbers). The mean rectal temperature is also shown (the vertical bars show the standard deviations).

**FIG. 3.** (A) Fecal excretion of S. dublin SVA47 after challenge of calves vaccinated with S. dublin SL7103 and rectal temperatures of the calves. (B) Fecal excretion of S. typhimurium SVA44 after challenge of calves vaccinated with S. dublin SL7103 and rectal temperatures of the calves.
At necropsy, calves from groups challenged with S. typhimurium SVA44 showed a purulent necrotizing panenteritis and atrophy of intestinal lymphatic tissues (not shown). The mucosa associated with the Peyer's patches in the terminal jejunum and the entire ileum was the most severely damaged. Purulent tonsillitis and acute hepatic degenerations were also seen.

In a bacteriological examination of specimens taken at necropsy, S. typhimurium SVA44 was recovered in more than 90% of the specimens.

**Immunologic examinations.** We estimated the delayed-type hypersensitivity to S. dublin and S. typhimurium crude antigens by intradermal injections of sonicated whole-cell suspensions of the SVA47 and SVA44 challenge strains. The calves were skin tested before and after either the second or third vaccine dose to minimize the immunizing effects of the skin testing. Nine of the 18 calves (3 from each group of 6) were tested 2 days before vaccination. None of the calves reacted with skin swellings exceeding the 4-mm increase in double skinfold thickness considered a positive reaction (Fig. 4). After two vaccinations (skin tested on day 12 and recorded on day 14), 7 of 12 calves given the S. dublin sonicate and 6 of 12 given the S. typhimurium sonicate showed positive skin reactions (most calves tested positive for both antigens). After three vaccinations, five of the six calves tested showed positive reactions to the S. dublin sonicate, but only three of six showed positive reactions to the S. typhimurium antigens. All six calves challenged with S. dublin SVA47 and skin tested 18 days after the infection (thus, three skin tests within 7 weeks) were positive to both the S. dublin and the S. typhimurium antigen preparations.

**Serum anti-LPS antibody titers.** The antibody titers for IgA, IgG1, IgG2, and IgM directed against S. dublin (O9,12) and S. typhimurium (O4,12) LPSs were estimated in an enzyme immunoassay (Table 1). In serum samples from the 18 calves immunized with SL7103, significantly increased IgM antibody titers directed against the S. dublin LPS were seen in samples from days 21 (0.001 < P < 0.01) and 28 (P < 0.001). Against the S. typhimurium LPS antigen, significant increases were seen in samples from day 14 on (days 14 and 21, 0.001 < P < 0.01; day 28, P < 0.001). After the challenge infection with S. dublin SVA47, all four antibody classes directed against the S. dublin and S. typhimurium LPSs were significantly elevated (Table 1).

In calves challenged with the lowest dose of S. typhimurium SVA44, which could be observed for only 7 days, significant increases were seen in IgA, IgG2, and, in particular, IgM against the S. typhimurium LPS antigen (Table 1).

**Immunohistopathological examinations.** Sections from all tissue specimens from the calves were examined by using the indirect PAP staining method. Two different mouse monoclonal antibodies were used, MASF O:9-1 specific for S. dublin and MAST O:4-2 specific for S. typhimurium. Both monoclonal antibodies reacted with the vaccine strain SL7103. The availability of two different O-antigen-epitope-specific antibodies made it possible to examine the tissues from challenged calves for the presence of both the vaccine, by using the O4-specific antibody on sections from S. dublin SVA47 (O9,12)-challenged calves and the O9-specific antibody on sections from S. typhimurium SVA44 (O4,5,12)-challenged calves, and challenge strains. We never found the vaccine strain in any tissue from any calf challenged with S. dublin SVA47 or S. typhimurium SVA44.

In the 12 calves challenged with S. typhimurium SVA44 (O4,5,12), O4-positive staining was seen in all layers of the intestinal mucosa and clusters were frequently found in degenerating foci in the mucosal propria (Fig. 5A) and in the lymphatic tissue of the Peyer's patches (Fig. 5B). Enterocytes were invaded, and cells containing PAP-positive material were markedly degenerated (Fig. 5C). At times, the villous layer appeared uninvaded and unaffected in spite of the high numbers of bacteria in the underlying degenerating tissues. High numbers of migrating leukocytes, mostly neutrophils, were seen in the mucosae of all affected parts of the intestine.

In the six calves challenged with S. dublin SVA47 (O9,12), O9-positive staining was found predominantly in the mucosal surface cells with few, or no, bacteria in the mucosal propria or lymphatic tissues (Fig. 5D). In addition, the S. dublin strain was found in the peribronchial glandular cells in the respiratory tract, the retropharyngeal glands, the biliary duct epithelium, the abomasal epithelium, and Brunner's glands in the duodenum, as observed in our previous study (20). This is in contrast to the localization of the S. typhimurium SVA44 strain (Fig. 5A to C).

**DISCUSSION**

The ΔaroA hybrid strain S. dublin SL7103, which besides its native O9,12 also expresses O4,12 of Salmonella serogroup B bacteria (16), was well tolerated by the 5- to 8-week-old calves when orally given in doses of from 10^9 (first dose) to 10^11 (third dose) live bacteria. Compared with its ΔaroA parent strain S. dublin SL5631 (which lacks the rfb chromosomal region of S. typhimurium and is recA1·), strain SL7103 caused a somewhat weaker febrile response and less diarrhea and was excreted in lower numbers and for a shorter period. The parent strain SL5631 was excreted in concentrations of ≥10^9 CFU/g for 7 days, whereas SL7103
was shed in the same concentration for only 1 day. However, SL7103 caused a vaccinemia in 5 of 18 calves and persisted in the calves for long periods because it could be recovered in enrichment broth culture for 7 days after oral vaccination.

The reason for the fewer adverse reactions and reduced excretion of SL7103 may be its recA1 mutation introduced into the strain to retain its partial diploid status with the rfb region of S. typhimurium. The recA1 mutation makes it recombination deficient and therefore unable to lose the duplicated segment. Strains made recA have been reported to grow less well than their parent strain. By performing comparative growth studies in BHI broth, we found that SL7103 grew at about the same rate, but optical density values of unshaken 37°C broth cultures reached plateau values ca. 0.2 unit lower and viable counts ca. fivefold lower than those for the recA+ strains (Fig. 1).

Calves vaccinated with SL7103 responded primarily with anti-S. dublin LPS IgM antibody titers which quantitatively and temporally were the same as those elicited by SL5631 (Table 1) (21). As estimated in the enzyme immunoassay, the anti-S. typhimurium LPS antibody titers were approximately the same as those seen against the S. dublin LPS (Table 1). The chemical analyses performed on LPS extracted from strain SL7103 showed (16) that the S. dublin and S. typhimurium specific repeating units were present in approximately the same proportions, and consequently we could expect antibody responses of the same magnitude. Challenge infection with virulent S. dublin SVA47 elicited high anti-S. dublin LPS antibody titers in the IgA, IgG1, IgG2, and IgM classes (Table 1). In the three calves surviving the S. typhimurium SVA44 challenge for 6 to 7 days, only a highly significant anti-S. typhimurium LPS IgM response was seen (Table 1).

In the delayed-type hypersensitivity skin tests, using crude whole bacterial homogenates (containing both LPS and outer membrane proteins including porins), almost as many calves given SL7103 responded as those given SL5631 (1). The increases in skinfold thickness were also almost the same, with no statistically significant (P > 0.05) differences observed. In responding calves, the skin reactivity against the S. typhimurium homogenate was of the same magnitude as that against the S. dublin homogenate (Fig. 4).

When SL7103-vaccinated calves were challenged with the virulent S. dublin SVA47 strain, we observed a protection that was as good as that in calves immunized with S. dublin SL5631 (21). This conclusion is based on observations of (i) the clinical well-being of the calves after challenge, (ii) the weak febrile response, (iii) the short excretion of SVA47, (iv) the inability to recover SVA47 from the tissues and intestine when the calves were sacrificed, and (v) the absence of any significant bacterial counts in the presence of only low numbers of S. dublin O-antigen-containing cells in the histopathological and immunohistochemical investigations. This suggests that the observation that SL7103 grew less than SL5631 in vitro had no influence on its activity to elicit protection against challenge with S. dublin SVA47.

In contrast to the excellent protection seen against S. dublin SVA47 challenge, no protection was seen against challenge with the virulent S. typhimurium SVA44. The first challenge dose chosen, ca. 3 x 10⁹ live bacteria (which corresponds to 10,000 LD₅₀), was approximately the same as that used in our previous study with the S. typhimurium SL1479 araA vaccine strain (3 x 10⁹ live bacteria) (19). After SL1479 vaccination, we observed a complete protection. However, in the present study, all six calves vaccinated with
FIG. 5. (A to D) Photomicrographs of PAP-stained tissue sections (positive complexes are stained brown). (A to C) Sections from a calf 6 days after oral infection with $5 \times 10^9$ live S. typhimurium SVA44 bacteria. (A) Ileum. The invading S. typhimurium SVA44 strain is visible in degenerating foci in the propria. (B) Ileum. The lymphatic aggregates of the Peyer's patches are degenerated, and PAP-positive Salmonella complexes are visible in marginal areas. (C) Jejunum. The villous architecture is destroyed, with S. typhimurium SVA44 visible in the degenerating superficial mucosa. (D) Section from the jejunum of a calf 6 days after an oral dose of $10^7$ live S. dublin SVA47 bacteria. The S. dublin strain is found almost exclusively in surface epithelial cells.
SL7103 had to be sacrificed within 48 h. A second group of calves was immunized with the same scheme but challenged with a 10-fold-lower dose (ca. $3 \times 10^8$ live *S. typhimurium* SVA44 bacteria) (Fig. 3B). Also with this challenge dose, the calves developed bacteremia and severe hemorrhagic enteritis and had to be sacrificed within 3 to 7 days. In addition, the challenge strain SVA44 was recovered from more than 90% of the specimens cultured, which illustrates that the calves suffered from and succumbed to a systemic infection.

It is evident that insertion of the *rfb* gene cluster specifying
the synthesis of the O-antigenic repeating unit of \textit{S. typhimurium} was insufficient to elicit a significant protective immunity in the calves. An \textit{S. typhimurium} anti-LPS response had been elicited by SL7103 as shown in the LPS enzyme immunoassay (absorption experiments demonstrated a specific anti-O4 response) and as suggested by the skin tests (Table 1; Fig. 4). Positive skin tests and protection from challenge exposure with \textit{S. typhimurium} were demonstrated previously in calves (17, 19). Apparently the host defense elicited by SL7103 was unable to control the \textit{S. typhimurium} SVA44 invasion of and multiplication in the tissues. It is possible that some, or complete, protection could have been provided with a lower challenge dose of SVA44. On the basis of our results, we feel safe in concluding that anti-LPS immunity alone will not protect the calf against high oral challenge doses. A further support for this conclusion was that parenteral immunization with an \textit{S. typhimurium} O-poly saccharide conjugate, which elicited high anti-O antigen titers, failed to protect calves against oral challenge with \textit{S. typhimurium} (unpublished data).

These data contrast with our own observations with NMRI mice and the intraperitoneal immunization and challenge model. In the mice we could elicit protection against at least 1,000 challenge LD50's with either \textit{S. dublin} SVA47 or \textit{S. typhimurium} SVA44 (16). We surmise that in the experimental mouse model with intraperitoneal immunization and challenge, antigens presented in the peritoneum, and antibodies playing a much more pronounced role than it does in the calf model with oral immunization and challenge and with invasion of the enterocytes (preferably in association with the Peyer's patches), intracellular replication, and spread to adjacent tissues, the lymphatics, and finally the blood.

An immune response to cell envelope porins has been reported to cause a high level of protection in mice (8, 13) between \textit{Salmonella} serogroups B and D as well. We were unable to observe any cross-protection in mice immunized intraperitoneally with the live \textit{aroA} mutant \textit{S. dublin} SL5631 and challenged with \textit{S. typhimurium} SVA44 (16). This could be interpreted as follows: (i) no antiporin immunity was elicited by the SL5631 immunization, (ii) the porin(s) in SVA44 was unaccessible to the antibodies, or (iii) there are antigenic differences between the porins in strains \textit{S. typhimurium} SVA44 and \textit{S. dublin} SL5631.

The immunohistochemical studies also revealed a striking difference between the tissue localization and damage caused by the virulent strains \textit{S. dublin} SVA47 and \textit{S. typhimurium} SVA44. The observations are based on studies of tissues from a total of 56 calves experimentally or naturally infected with either SVA47 or SVA44 calves experimentally vaccinated with the live \textit{aroA} auxotroph \textit{S. dublin} SL5631 or \textit{S. typhimurium} SL1479. A uniform picture emerged. The \textit{S. dublin} strain was found predominantly in the columnar enterocytes of the terminal jejunum (Fig. 5D) and ileum and in the follicle-associated epithelium over the Peyer's patches, but it was also found in the glandular tissues in the duodenum, the tonsillar area, and the lungs (20). In contrast, the \textit{S. typhimurium} strain was found in all layers of the intestinal mucosa; clusters were frequently found in degenerating foci in the mucosal propria (Fig. 5A) and in the lymphatic tissue of the Peyer's patches (Fig. 5B). We sometimes observed an apparently unaffected villous layer overlying invaded degenerated submucosal tissues. At present, we are unaware of whether these differences are particular to strains \textit{S. dublin} SVA47 and \textit{S. typhimurium} SVA44 or whether they are characteristic of the serovars. In any case, it is obvious that there are different modes of tissue dissemination and destruction between SVA44 and SVA47, which most likely have their explanation in the different virulence factor(s). Understanding such differences may be of importance for the construction of efficacious vaccines.

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REFERENCES

aroA O4-O9 SALMONELLA VACCINE IN CALVES


