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Antibody Response and Protection against Challenge in Mice Vaccinated Intraperitoneally with a Live *aroA* O4-O9 Hybrid *Salmonella dublin* Strain

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An auxotrophic *Salmonella dublin* (O9,12) strain, SL5631, with a deletion affecting gene *aroA*, was made into a partial diploid expressing the *rfb* (O-antigen-repeat-unit-specifying) gene cluster of *Salmonella typhimurium* (O4,12). By use of O4- and O9-specific antisera in indirect immunofluorescence assays, the resulting hybrid SL7103 was shown to express both the O4- and O9-antigen epitopes in the same bacterium. Qualitative and quantitative sugar analyses by gas-liquid chromatography on peralditol acetates of phenol-water-extracted lipopolysaccharides showed that the *S. dublin* and *S. typhimurium* repeating units (estimated on the basis of their tyvelose and abequose contents, respectively) were present in approximately equimolar amounts. The SL7103 hybrid auxotroph was avirulent when given intraperitoneally to NMRI mice in a dose of 10^8 CFU and elicited a protective immunity against intraperitoneal challenge with either virulent *S. dublin* (50% lethal dose of ca. 1.5×10^4 CFU versus $<1 \times 10^1$ CFU in nonimmunized mice) or virulent *S. typhimurium* (50% lethal dose of ca. 1×10^5 versus $<1 \times 10^1$ CFU in nonimmunized mice). Compared with the protection elicited in homologous systems (*S. dublin* SL5631 against *S. dublin* and *S. typhimurium* SL1479 against *S. typhimurium*), the protective efficacy of the hybrid was reduced approximately 70-fold against *S. dublin* challenge and 100-fold against *S. typhimurium* challenge. Vaccination with *S. typhimurium* SL1479 conferred no protection against *S. dublin* challenge, and vaccination with *S. dublin* SL5631 conferred no protection against *S. typhimurium* challenge. The protection elicited by the hybrid strain SL7103 is supposed to be mainly a consequence of serum antibodies directed against the immunodominant O4 and O9 epitopes.

Salmonellosis is still a major disease in cattle, and infections caused by *Salmonella dublin* (O9,12) and *Salmonella typhimurium* (O4,5,12), in particular, are common all over the world (9, 37). Attempts to control *Salmonella* infections by hygienic measures, e.g., quarantine, restricted contacts, and slaughter of infected herds, etc., have been only marginally successful.

There has recently been much interest in other measures for control such as active immunization (9, 37). Advances in molecular biology have made it possible to introduce defined, nonrevertible lesions in the chromosome, resulting in nonvirulent strains or strains unable to grow in the absence of certain metabolites (6, 10, 21, 32).

Salmonella vaccines confer substantial protection against later challenge with virulent strains of corresponding serotypes. Soon after vaccination with killed and live vaccines, a degree of cross-protection between different *Salmonella* O serotypes can be seen, but later protection seems to be O-antigen specific (8, 12). A *Salmonella* live vaccine strain able to express both O4 and O9 antigens might be useful for protection of calves against both *S. dublin* (O9,12) and *S. typhimurium* (O4,5,12) infection. Hybrid *Salmonella* strains, e.g., *S. typhimurium* expressing O9 antigen, have been constructed by conjugation (14, 34). Since they were the result of allelic exchange, the O4 specificity was replaced by O9 specificity, and consequently the hybrids failed to express both O antigens simultaneously. We recently described the construction and characterization of *Salmonella* hybrids expressing both O4 and O9 (13, 35). The recipient strains in these studies, *S. typhimurium* LT2 and mouse-virulent *Salmonella enteritidis* SL5603, were considered less suitable as hosts for later vaccine studies in calves. A calf-virulent Swedish strain *S. dublin* SVA47 was therefore used as a host. It was made auxotrophic by transduction of *ΔaroA148*, a spontaneous deletion mutation, resulting in strain SL5631 (29).

In this communication we describe the construction of a stable O4-O9 hybrid strain derived from SL5631 which simultaneously expresses O9,12 of *S. dublin* and O4,12 of *S. typhimurium*. The immune response in and protection of NMRI mice given strain SL7103 as a live vaccine intraperitoneally and challenged intraperitoneally with either virulent *S. dublin* or virulent *S. typhimurium* are described. The results are compared with those of NMRI mice given either *S. dublin* SL5631 or the *aroA* live strain *S. typhimurium* SL1479 (10, 25, 30) and the newly constructed SL7368.

MATERIALS AND METHODS

Experimental animals. Health-controlled NMRI mice (SVA, Uppsala, Sweden), male and female, 7 to 9 weeks old, weighing 20 to 25 g, were used throughout the study. The mice were kept in cages, with 5 or 10 animals in each, and allowed free access to commercial food and water.

Bacterial strain construction. The starting strains and derivatives constructed from them are listed in Table 1. The procedures for selection of the transductants with tetracycline resistance, $SerC^+$ (i.e., serine and pyridoxine independent), or $HisD^+$ (able to utilize histidinol as the sole source

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TABLE 1. Bacterial strains

Strain	Description[a]	Source or reference[b]
SVA47	*S. dublin* wild type, virulent	National Veterinary Institute strain collection
SL5621	*S. dublin* SVA47 *aroA(serC)1121*::Tn*10*	From SVA47 by transduction
SL5631	*S. dublin* SVA47 *serC*$^+$ Δ*aroA148*	From SL5621 by transduction
SL5814	*S. dublin* S4454 *srl-2*::Tn*10 recA1*	Stocker laboratory stock
SL5896	*S. dublin* SVA47 Δ*aroA148 gnd*::Tn*10* Δ*his-203*	From SL5631 by transduction
SL5898	*S. dublin* SVA47 Δ*aroA148* Δ*his-203* CRR[*gnd*::Tn*10*(Tc^s)]	From SL5896 by selection for tetracycline sensitivity
SL7102	*S. dublin* SVA47 Δ*aroA148* Δ*his-203* CRR[*gnd*::Tn*10*(Tc^s)] "pi-422" (i.e., with tandem duplication causing HisD$^+$ phenotype and O4 characteristic because of inclusion of group B *rfb*$^+$ gene cluster)	From SL5898 by transduction with selection for histidinol utilization
SL7103	*S. dublin* SVA47 Δ*aroA148* Δ*his-203* CRR[*gnd*::Tn*10*(Tc^s)] "pi-422" *srl*::Tn*10 recA1*	From SL7102 by transduction
aroA148	*S. typhimurium* LT2 with extensive deletion at *aroA*	Nishioka et al. (20); Halula and Stocker (9a)
his-203	*S. typhimurium* LT2 with deletion of promoter and proximal structural gene, *hisG*, of *his* (histidine biosynthesis) operon	Anderson and Roth (1)
NK114	*S. typhimurium* LT2 *edd gnd*::Tn*10*	N. Kleckner
SL1479	*S. typhimurium* 108-11 CRR426[*aroA544*::Tn*10* (Tc^s, non-rev)] live-vaccine strain, O1,4,12	Smith et al. (30)
SL5340	*S. typhimurium* LT2 Δ*his-203 gnd*::Tn*10*	From Δ*his-203* by transduction; Johnson et al. (13)
SL7363	*S. typhimurium* SVA44 *aroA(serC)1121*::Tn*10*	From SVA44 by transduction
SL7368	*S. typhimurium* SVA44 *serC*$^+$ Δ*aroA148*	From SL7363 by transduction
SVA44	*S. typhimurium* wild type, virulent; O4,5,12	National Veterinary Institute strain collection
TR5214	*S. typhimurium* LT2 Δ*his-203* "pi-422" (i.e., with tandem duplication causing HisD$^+$)	Anderson and Roth (1)
TT472	*S. typhimurium* LT2 *aroA(serC)1121*::Tn*10*	J. Roth; Hoiseth and Stocker (11); Edwards and Stocker (7)

[a] Nonrelevant characteristics of some strains are not listed. Some gene symbols are shortened after first mention.
[b] See Materials and Methods for transductional donors.

of histidine) are as described previously (7, 10, 11, 13). The two virulent strains, *S. typhimurium* SVA44 and *S. dublin* SVA47, used both as challenge and as parents of Δ*aroA* strains SL5631 and SL7368, respectively, are from the strain collection of the Department of Bacteriology, National Veterinary Institute, Uppsala, Sweden. To obtain a nonreverting aromatic-dependent candidate live-vaccine strain from *S. dublin* SVA47, this strain was first given insertion mutation *aroA*(*serC*)*1121*::Tn*10* by transduction from *S. typhimurium* TT472 and with selection for tetracycline resistance. This mutation, inferred to consist of insertion of transposon Tn*10* in the promoter-proximal gene *serC* or in the promoter region of the *serC-aroA* operon (11), causes a SerC$^-$ Aro$^-$ phenotype that is a requirement for serine plus pyridoxine and for several aromatic metabolites. A transductant with the new auxotrophic characteristics in addition to a parental requirement for nicotinic acid, described as SL5621, was used as the recipient in a second step of transduction in which the donor was *S. typhimurium* LT2 *aroA148*, a strain inferred from genetic analysis (20) to have an extensive deletion in gene *aroA*. This strain was recently shown, by failure to bind a DNA probe, to lack at least the ca. 600 C-terminal base pairs of gene *aroA* (9a). Selection was made for the SerC$^+$ characteristic on defined medium supplemented with an aromatic cocktail and nicotinic acid. Most of the transductant clones obtained were still aromatic dependent but tetracycline sensitive, as expected from replacement of *aroA*(*serC*)*1121*::Tn*10* by *serC*$^+$ *aroA148* of the donor. One such strain was named SL5631. A Δ*aroA148* derivative, SL7368, was similarly made by two steps of transduction from the Swedish *S. typhimurium* challenge strain SVA44. Strain SL5631 (same as *S. dublin* SVA47 made Δ*aroA148*) was then made into a partial diploid, with a tandem duplication of a chromosomal segment including the *his* biosynthesis operon and the *rfb* (O-repeat-unit-specifying) gene cluster, by a modification of the procedure of Johnson et al. (13). In the resulting strain, the original chromosomal copy of the *rfb* gene cluster, of *S. dublin* origin, causes production of O repeat units with a tyvelose branch and therefore is of antigenic character O9; the second copy, of *S. typhimurium* origin, codes for the production of abequose-containing units and therefore is of antigenic character O4. (Factor O5 of the *S. typhimurium* donor is not expressed in the partial diploid strain because the gene for the abequose acetylation and thus for O5 expression is not included in the transduced chromosome fragment that includes the join-point whose insertion in the recipient chromosome generates a duplication of a segment of it [1, 13].) To obtain a partial diploid derivative of strain SL5631, it was first made Δ*his-203* by cotransduction with *gnd*::Tn*10* and selection for tetracycline resistance; the strain used as a donor, SL5340, was made *gnd*::Tn*10* by transduction from strain NK114, LT2 *edd gnd*::Tn*10*. The *his-203* deletion removes the promoter and the proximal structural gene, *hisG*, of the histidine biosynthesis operon but leaves intact the *hisD* and the distal *his* biosynthesis genes; some mutants obtained from *his-203* by selection for the ability to utilize histidinol as a source of histidine (that is, for recovery of HisD function) arise by generation of a tandem duplication with a join-point upstream from gene *hisD*, placing it (and the rest of the operon) under the control of an unrelated, appropriately oriented promoter (1). These histidinol-responding mutants remain auxotrophic because of the absence of *hisG* function. Strain SL5631 (same as *S. dublin* SVA47 *aroA148*) made *gnd*::Tn*10 his-203* was named SL5896. A tetracycline-sensitive mutant of it (isolated by the procedure of Bochner et al. [3]) was named SL5898. To introduce a tandem duplication known to include the *his* and

rfb gene clusters into strain SL5898, this latter strain was treated with phage P22 HT105/1 *int* grown on strain TR5214, a histidinol-utilizing tandem-duplication mutant of *his-203*. Histidinol-utilizing transductants were selected on defined medium supplemented with nicotinic acid (required by *S. dublin* SVA47 and most other strains of this serotype), an aromatic cocktail (10), because of its *aroA148* characteristic, and histidinol, at 150 μg/ml, which is needed to obtain reasonably rapid growth with histidinol as the sole source of histidine; in the hope of accelerating the growth of $HisD^+$ transductant colonies, the selective medium was further supplemented with a small amount of histidine assay medium (Difco). A transductant found to be O4-O9 by slide agglutination test was named SL7102. It was treated with a lysate of strain SL5814, which is *S. dublin srl-2*::Tn*10 recA1*. A tetracycline-resistant transductant inferred from its sensitivity to UV irradiation to be *recA1* was named SL7103.

S. typhimurium SL1479 is a nonreverting aromatic-dependent tetracycline-sensitive mutant of an *aroA544*::Tn*10* derivative of a wild-type calf-virulent strain of *S. typhimurium*, UCD108-11; its construction and trials as a live vaccine in calves have been described (25, 30). SL7368 is the Swedish *S. typhimurium* challenge strain SVA44 made Δ*aroA148*, as described above.

Salmonella thompson IS40 (O6,7) was from the strain collection of the Department of Clinical Bacteriology, Huddinge Hospital.

Immunization and challenge. Vaccine and challenge strains were grown overnight on nutrient agar plates (Difco) and then inoculated into brain heart infusion broth (Difco B37) and incubated unshaken at 37°C for 18 h. The growth was adjusted to approximately 10^9 CFU/ml by dilution with phosphate-buffered saline (PBS; pH 7.4), after measurement of A_{595}. The number of CFU per milliliter was determined by plate count. For vaccination, all cultures were diluted in PBS to 1×10^6 CFU, and 0.2 ml (i.e., 2×10^5 CFU) was given intraperitoneally through a 0.6-mm cannula on days 0, 7, and 14. The 50% lethal doses (LD_{50}) for the challenge and vaccine strains were determined by inoculation of groups of 10 NMRI mice, with each group given from $\sim10^9$ to $\sim10^1$ CFU in 10-fold dilution steps in a volume of 0.2 ml. The challenge dose was given on day 28, i.e., 14 days after the final vaccine dose. Mice were inspected twice daily, and apparently moribund mice were sacrificed. The LD_{50} calculations were done by the method of Reed and Muench (22). Sacrificed mice were examined for the presence of either the vaccine or the challenge strain in the intestine, liver, and spleen. The organs were removed aseptically and mechanically homogenized in 1.0 ml of PBS, and 0.1-ml samples of dilutions in PBS were streaked on nutrient agar (Difco) and incubated at 37°C overnight. The rest of the homogenate was enriched in 5.0 ml of selenite broth (Difco) at 37°C for 18 h, plated on brilliant green agar (Difco), and incubated at 37°C for 18 h. Suspect *Salmonella* colonies were identified by biochemical and serological tests.

LPS extraction and sugar analyses. The bacteria were grown in submerged culture, and the lipopolysaccharide (LPS) was extracted with hot phenol-water as described previously (16). The sugar analysis was performed basically by the method of Sawardeker et al. (26). In brief, the *Salmonella* SL7103 LPS was hydrolyzed with 0.5 M trifluoroacetic acid at 100°C for 16 h. After reduction with sodium borohydride (10 mg/ml in 1 M NH_3 for 2 h at 25°C), the sugar alditols were converted to their corresponding acetates with acetic acid anhydride in the presence of pyridine. The alditol acetates were separated by using a Hewlett-Packard gas chromatograph (model 5890; Palo Alto, Calif.) on a DB-225 fused silica capillary column (30 m by 0.25 mm) (J & W Scientific, Folsom, Calif.) at 220°C. The sugar derivatives were identified by using authentic standards. For quantitative analysis, xylose was used as an internal standard.

Indirect immunofluorescence. The technique and the antisera and conjugates used have been described before (35). Briefly, one colony of the *Salmonella* strain to be examined was suspended in PBS, and 20-μl aliquots were applied to a glass slide. After drying and heat fixation, the film was covered with either rabbit anti-abequose-α1,3-mannose-bovine serum albumin immunoglobulin G (IgG) (O4 specific) (diluted 1/40 in PBS), mouse monoclonal antibody MASE 9-1 (O9 specific) (diluted 1/100 in PBS), or a mixture of the O4 and O9 antibodies. In other experiments, the strains were tested with the rabbit anti-tyvelose-α1,3-mannose-bovine serum albumin IgG (O9 specific) (diluted 1/40 in PBS) and the mouse monoclonal antibody MAST 4-2 (O4 specific) (diluted 1/80 in PBS). After incubation at 22°C for 30 min, the slides were rinsed three times with PBS for 10 min. Bound rabbit antibodies were detected with swine anti-rabbit tetramethyl-rhodamine-isothiocyanate-conjugated immunoglobulin (diluted 1/50 in PBS) (Dakopatts Hägersten, Sweden). Bound mouse monoclonal antibodies were detected with goat anti-mouse fluorescein-isothiocyanate-conjugated whole IgG molecules (diluted 1/20 in PBS) (Sigma Chemical Co., St. Louis, Mo.). The incubations with conjugates were performed at 22°C for 30 min. The slides were rinsed as described above and subsequently mounted with 90% buffered glycerol (pH 8.2). All incubations were performed in a moist chamber. The *S. thompson* IS40 (O6,7) strain served as a control for nonspecific staining.

Slides were examined in a Nikon Labophot fluorescence microscope with incident light by using an HBO 100 mercury lamp as the light source. The microscope was equipped with filter combination B2A (450 to 490 nm with a barrier filter at 520 nm) for fluorescein isothiocyanate visualization and with filter combination G2A (510 to 560 nm with a barrier filter at 590 nm) for tetramethyl-rhodamine-isothiocyanate visualization. There was only a weak, and insignificant, spillover of tetramethyl-rhodamine-isothiocyanate into the fluorescein isothiocyanate range, giving a faint reddish staining of the bacteria.

EIA. Blood samples, approximately 0.3 ml from each mouse, were taken from the retro-orbital plexus of ether-anesthetized animals on days 0 (prebleed), 7, 14, 28, 35, 42, and 58 of the experiment. All sera from the 10 mice of each group were pooled and kept frozen at −20°C until analyzed. The enzyme immunoassay (EIA) was performed by the method of Engvall and Perlmann, modified for polystyrene microtiter plates (AS Nunc, Roskilde, Denmark) as previously described (15). The wells were coated with 100 μl of LPS antigen (10 μg of phenol-water-extracted *S. dublin* SVA47 [O9,12], *S. typhimurium* SH4809 [O4,5,12] [25], or *S. thompson* IS40 [O6,7] [25] per ml in 0.05 M carbonate buffer [pH 9.6]) at 20 to 25°C for 18 h. Control wells were treated with coating buffer only. Before use, the plates were washed three times with washing buffer, 0.15 M NaCl containing 0.05% (vol/vol) Tween 20. The sera to be assayed were diluted 1:1,000 in incubation buffer PBS-T (PBS [pH 7.4] containing 0.05% [vol/vol] Tween 20). Aliquots (100 μl) of diluted sera to be tested, one positive and one negative control serum, and incubation buffer only, all in duplicate, were added to the microtiter plates. These were incubated at 22°C for 2 h and washed as described before. For class-specific titer determinations, alkaline phosphatase-conju-

gated goat anti-mouse IgG, IgM (Jackson Immunoresearch Lab Inc., West Grove, Pa.), and IgA (Zymed Laboratories, South San Francisco, Calif.) sera (100 μl), diluted in PBS-T, were then added to the wells, and the microtiter plates were incubated at 22°C for 18 h. For other titer determinations, an alkaline phosphatase-conjugated sheep anti-mouse immunoglobulin serum (Sigma) was used. After being washed, the wells were filled with 100 μl of enzyme substrate solution (paranitrophenyl phosphate [1 mg/ml] in 1.0 M diethanolamine-HCl buffer [pH 9.8] containing 0.5 mM $MgCl_2$) and incubated. The A_{405} was determined in a Titertek Multiscan photometer (Flow Laboratories Ltd., Irvine, Scotland) after 25, 50, and 100 min. The A_{405} seen in control wells was uniformly less than 0.100 at 100 min and was not accounted for. The intra-assay variation was less than ±5%, and the interassay variation was less than ±15%, as estimated with the positive and negative control sera. The relative titers given are the absorbance values multiplied by the dilution factor (1,000).

RESULTS

Construction and characterization of *S. dublin* SL7103. The construction of an *aroA* deletion derivative of *S. dublin* SVA47 (see Materials and Methods) was achieved without difficulty when allowance was made for the very slow growth of the *aroA148* class on the supplemented minimal medium used to select for $SerC^+$ transductants. The introduction of the *his-203* deletion by cotransduction with *gnd*::Tn*10* was also successful, although the number of transductant colonies obtained in this and other crosses of *Salmonella* strains of O group B as the donor with strains of O group D as the recipient was low, perhaps because of partial genetic nonhomology or DNA restriction. All of several *gnd*::Tn*10* *his-203* transductant clones tested were $O9^+$ $O4^-$ by slide agglutination tests, showing nonreplacement of the recipient group D rfb^+ gene cluster by group B rfb^+ of the donor, despite fairly close linkage (with gene order *his-gnd-rfb*). Tetracycline-sensitive mutants of SL5896 (same as *S. dublin* SVA47 *aroA148 his-203 gnd*::Tn*10*) were obtained by selection for tetracycline-sensitive mutants on the autoclaved chlortetracycline-fusaric acid medium (3). This medium was supplemented with 2,3-dihydroxybenzoic acid to allow growth of the aromatic-dependent mutant (*aroA148* defect) and synthesis of enterochelin needed to capture iron. Introduction of a chromosomal segment including the join-point of the tandem duplication of strain TR5214 was achieved by selection for the ability to utilize histidinol (supplied at 150 μg/ml) as a source of histidine. Very small colonies of $HisD^+$ transductants were seen after several days of incubation at 37°C. In one experiment, 1 of 13 such colonies, tested after single-colony reisolation on the selective medium, reacted by slide agglutination with both O4 and O9 sera; the other 12 clones failed to agglutinate in anti-O4 serum and are inferred to result from incorporation of the join-point (and immediately adjacent *his* operon) but not of the less-close group B rfb^+ gene cluster.

The O4-O9 transductant strain retained for further investigation, SL7102, was, as expected, unstable, losing its $HisD^+$ and O4-O9 characteristics at high frequency, probably by the recombination or slipping during chromosome replication of the two copies of the duplicated segment. For instance, after a single passage in broth without selection for the $HisD^+$ characteristic, 22 of 40 colonies from streaking on rich medium failed to react with anti-O4 serum. To stabilize its partially heterozygous state, strain SL7102 was treated with phage grown on an *S. dublin* strain which is *srl-2*::Tn*10* *recA1*; some tetracycline-resistant clones showed the UV sensitivity expected of a *recA* mutant. One such transductant, named SL7103, was stable in the O4-O9 $HisD^+$ state and was used as live vaccine.

Strain SL7103 showed a variable degree of reaction with anti-O1 serum; in *S. typhimurium*, the presence of factor 1, because of a glucosyl attached α1→6 to the galactose of the basic trisaccharide O repeat unit, almost always results from lysogeny or infection with a converting phage of the A1-A2 group (31). However, phage P22.c2 was active on the O4-O9 *recA* strain, indicating nonlysogeny and absence of free phage P22 HT105/1 *int*. (All of a group of *S. dublin* wild-type isolates tested gave positive slide agglutination, sometimes very weak, when tested with Difco anti-O1 serum but did not release any phage detectable by plaque formation on the indicators tested.)

The nonreverting aromatic-dependent and histidine (or histidinol)-requiring strain with tandem duplication stabilized by the *recA* mutation was confirmed as smooth as judged by the pattern of sensitivity to a collection of smooth-specific and rough-specific phages (36).

The simultaneous expression of both the *S. dublin* (O9,12) and *S. typhimurium* (O4,12) O antigens in strain SL7103 was studied by using immunofluorescence with a mixture of rabbit anti-α-tyvelose-1→3α-mannose-1-bovine serum albumin (O9-specific) serum and an O4-specific mouse monoclonal antibody or in other tests with a mixture of rabbit anti-α-abequose-1→3-mannose-1-bovine serum albumin (O4-specific) antiserum and an O9-specific mouse monoclonal antibody (35). By using fluorescein isothiocyanate-conjugated anti-rabbit and tetramethyl-rhodamine-isothiocyanate-conjugated anti-mouse antisera, we could observe the simultaneous expression of the O9 and O4 epitopes in virtually all SL7103 bacteria (figures not shown). SL7103 bacteria were negative when incubated with the conjugated secondary antibodies only. No nonspecific staining was observed with any antibody-secondary antibody combinations when *S. thompson* (O6,7) IS40 was used as the control.

A chemical proof of the expression of O4 and O9 was obtained by growing SL7103 in submerged culture and extracting LPS with hot phenol-water. A qualitative and quantitative sugar analysis of the LPS by gas-liquid chromatography of peracetylated alditol acetates showed the presence of rhamnose, mannose, and galactose in the expected ratio of 1:1:1. The ratios of abequose/tyvelose were 0.54:0.46 from one batch of bacteria and 0.57:0.43 from another (Fig. 1). The amounts of the dideoxyhexosyls were somewhat smaller than those of rhamnose and the hexoses since no precautions were taken to prevent a partial destruction during hydrolysis. The results indicate a stable expression of both repeating units in the O-antigenic polysaccharide chains of SL7103, with a slight dominance of that from *S. typhimurium* (O4 epitope containing repeating unit).

Protection of intraperitoneally vaccinated NMRI mice. One group of 40 NMRI mice was given a single intraperitoneal dose of 2×10^5 CFU of SL7103, and another group received a single dose of 2×10^7 CFU. Ten mice were sacrificed from each group on days 7, 14, 21, and 28, and their livers, spleens, and intestines were cultured for the presence of the SL7103 vaccine strain. The bacterial counts were low from the tissues, and in most instances the vaccine strain was recovered only after enrichment culture. Therefore, the data are given as the number of mice with growth of SL7103. In mice given 2×10^5 CFU in a single dose, SL7103 was recovered from 5 of 10 livers on day 7 and from 1 of 10 livers

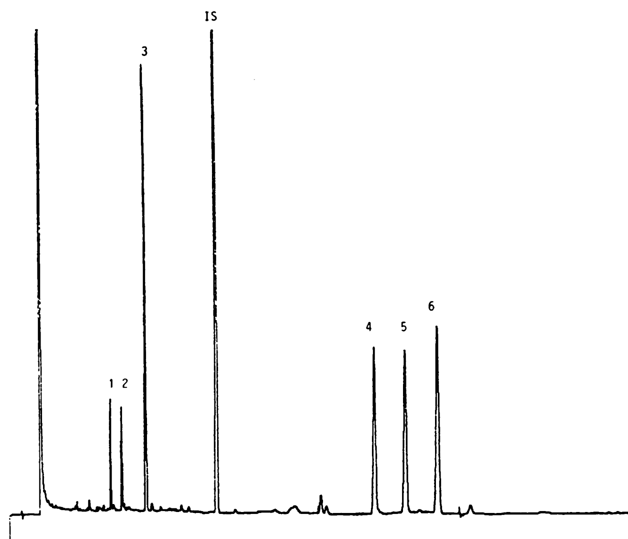

FIG. 1. Gas chromatogram of alditol acetates obtained from the *S. dublin* O4-O9 strain SL7103 LPS. IS, internal standard (xylose). Peaks: 1, abequose; 2, tyvelose; 3, L-rhamnose; 4, D-mannose; 5, D-galactose; 6, D-glucose.

on each of days 14 and 21 (Fig. 2). SL7103 was recovered from 4 of 10 spleens only on day 7. In mice given 2×10^7 CFU in a single dose (not shown in Fig. 2), almost all organs yielded growth of SL7103 on day 7. On day 14, SL7103 was recovered from 4 of 10 livers and 5 of 10 spleens. On day 21, SL7103 was recovered from a single spleen, but on day 28 all cultures were negative for SL7103.

In mice given 2×10^5 CFU SL7103 could not be recovered from a single intestine. With the higher dose of 2×10^7 CFU, 6 of 10 intestines yielded growth of SL7103 on day 7. Thereafter, all intestines were negative.

In a subsequent experiment, 100 NMRI mice were vaccinated intraperitoneally with a single dose of 2×10^5 CFU of SL7103 and another group of 100 mice were vaccinated with a single dose of 2×10^7 CFU. They were challenged intraperitoneally on day 21: groups of 10 mice were challenged with $\log_{10}$ dilutions of virulent *S. dublin* SVA47 or *S. typhimurium* SVA44. The single-dose vaccination of 2×10^7 CFU of SL7103 gave only moderate protection against both strains, with an LD_{50} of 10^2 against both SVA44 and SVA47 (data not shown). The single dose of 2×10^5 CFU gave no protection, with an LD_{50} for both strains of ~10 (data not shown). On the basis of these experiments, we decided to vaccinate with a dose of 2×10^5 CFU on days 0, 7, and 14. In a pilot study, 50 NMRI mice were vaccinated, groups of 10 mice were sacrificed on days 7, 14, 21, 28, and 35, and their livers, spleens, and intestines were cultured for persistence of the vaccine strain (Fig. 2). The number of organs with growth of SL7103 declined over time: on day 21 (1 week after the third vaccination), 3 of 10 livers and spleens showed growth of SL7103; however, on days 28 and 35, SL7103 could no longer be recovered from any organ. SL7103 was grown from the intestines of two mice on day 7 and of one mouse on day 14. On the basis of these results, the subsequent studies were done with mice given three intraperitoneal vaccinations with 2×10^5 live bacteria on days 0, 7, and 14, with challenge on day 28.

The strains used as vaccines were *S. dublin* SL5631 *aroA* (O9,12), *S. typhimurium* SL1479 and SL7368 *aroA* (O4,5,12), and *S. dublin* SL7103 *aroA* (O9,12; O4,12). The mice were challenged intraperitoneally with graded doses of 10^1 to 10^7 CFU of either of the virulent strains *S. dublin* SVA47 and *S. typhimurium* SVA44 (Table 2).

Mice vaccinated with *S. dublin* SL5631 were protected against challenge with its virulent parent strain *S. dublin* SVA47: the LD_{50} was 10^6 CFU of SVA47 for vaccinated mice as compared with $<10^1$ CFU of SVA47 in the nonvaccinated control group (Table 2; Fig. 3A). Vaccination with *S. dublin* SL5631 did not, however, result in any significant protection against challenge with *S. typhimurium* SVA44: the LD_{50}s in vaccinated and nonvaccinated mice were ≈10 and <10 CFU of SVA44, respectively (Table 2; Fig. 3B).

Mice vaccinated with live *S. typhimurium* SL1479 were protected against challenge with virulent *S. typhimurium*

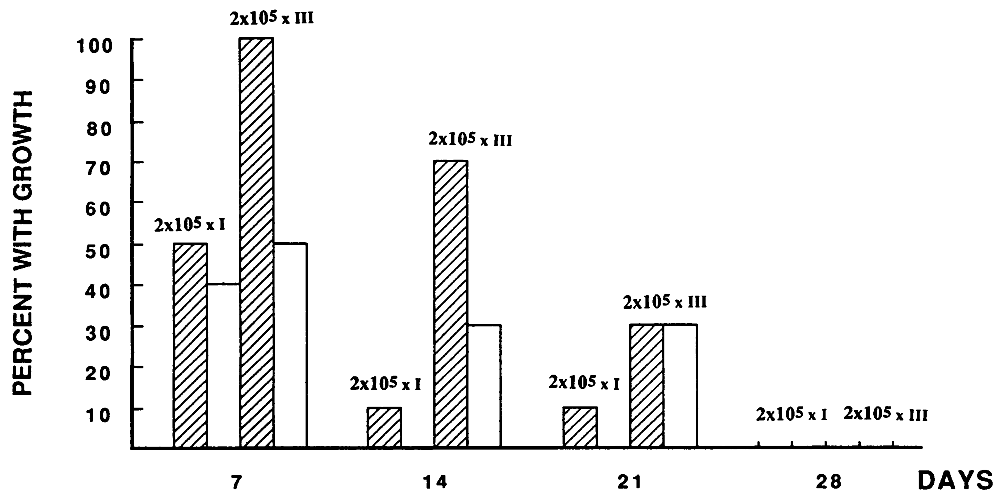

FIG. 2. Percent mouse livers (hatched bars) and spleens (open bars) from which the *S. dublin* O4-O9 hybrid strain SL7103 was recovered. Forty mice were vaccinated intraperitoneally with 2×10^5 CFU on day 0, and groups of 10 mice were sacrificed on days 7, 14, 21, and 28. Another group of 50 mice was vaccinated intraperitoneally with 2×10^5 CFU on days 0, 7, and 14. Groups of 10 mice were sacrificed on days 7, 14, 21, 28, and 35. Roman numerals indicate the number of vaccine doses given.

TABLE 2. Protection measured as LD_{50} against intraperitoneal challenge with virulent *S. dublin* SVA47 or *S. typhimurium* SVA44 in mice intraperitoneally vaccinated with live attenuated *S. dublin*, *S. typhimurium*, or *S. dublin* O4-O9 hybrid vaccine strains

Vaccine[a]	Challenge[b]	LD_{50}[c] (CFU)
None	*S. dublin* SL5631	$>1 \times 10^8$
	S. typhimurium SL1479	$>1 \times 10^8$
	S. typhimurium SL7368	$>1 \times 10^8$
	S. dublin O4-O9 SL7103	$>1 \times 10^8$
	S. dublin SVA47	$<1 \times 10^1$
	S. typhimurium SVA44	$<1 \times 10^1$
S. dublin SL5631	*S. dublin* SVA47	1×10^6
	S. typhimurium SVA44	1×10^1
S. typhimurium SL1479	*S. dublin* SVA47	1.5×10^1
	S. typhimurium SVA44	1×10^7
S. typhimurium SL7368	*S. typhimurium* SVA44	1×10^4
S. dublin O4-O9 SL7103	*S. dublin* SVA47	1.6×10^4
	S. typhimurium SVA44	1×10^5

[a] Live vaccine in a dose of ca. 2×10^5 CFU was given on days 0, 7, and 14.
[b] The live challenge in $\log_{10}$ dilution steps was given intraperitoneally on day 28.
[c] Mice were observed for up to 30 days after challenge. The LD_{50}s were calculated by the method of Reed and Muench (22).

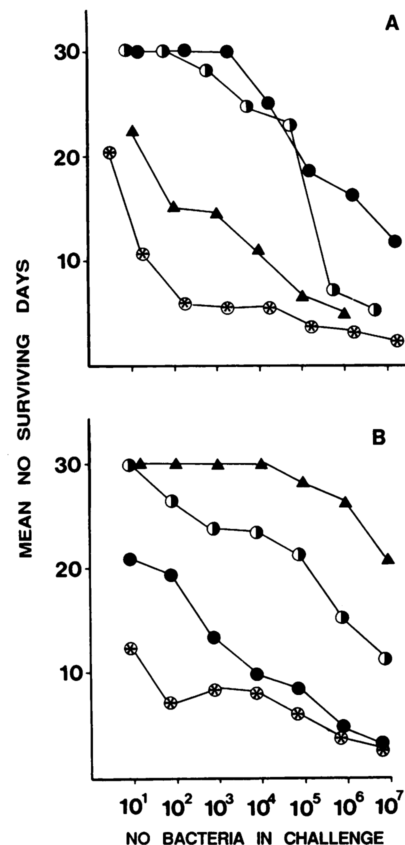

FIG. 3. Mean number of days of survival in NMRI mice challenged intraperitoneally with graded doses of *S. dublin* SVA47 (A) or *S. typhimurium* SVA44 (B). Symbols: ⊛, nonvaccinated mice; ▲, vaccinated intraperitoneally with *S. typhimurium* SL1479; ●, vaccinated intraperitoneally with *S. dublin* SL5631; ◑, vaccinated intraperitoneally with the *S. dublin* O4-O9 hybrid strain SL7103.

SVA44, with LD_{50}s of 10^7 CFU in vaccinated mice and $<10^1$ CFU in nonvaccinated mice (Table 2). However, no significant protection was elicited against challenge with *S. dublin* SVA47, with an LD_{50} of ≈15 CFU as compared with an LD_{50} of <10 CFU for nonvaccinated mice. The newly constructed *S. typhimurium* SL7368, which is *S. typhimurium* SVA44 with a deleted *aroA*, was 1,000-fold less efficient as a vaccine than SL1479 in eliciting a protective efficacy against SVA44 challenge. The reason(s) for the observed difference in protective efficacy between the two *S. typhimurium aroA* vaccines remains unknown.

For vaccination with the hybrid *S. dublin* strain SL7103, which expresses the O4-O9,12 antigens, groups of 20 mice were used. Ten mice from each group were challenged with *S. dublin* SVA47, and 10 were challenged with *S. typhimurium* SVA44. The vaccination elicited protection against each challenge: the LD_{50}s were 1.6×10^4 for *S. dublin* SVA47 and 1×10^5 for the *S. typhimurium* SVA44 challenge (Table 2). This means that the immunization increased the LD_{50} by >1,000-fold for each of the two virulent challenge strains. Although the protection is highly significant ($P < 0.001$), it is still 1.8 log units lower than the protection elicited with *S. dublin* SL5631 and 2 log units lower than the protection elicited with *S. typhimurium* SL1479 (but 1 log unit higher than that of *S. typhimurium* SL7368, the *aroA* auxotroph of SVA44).

The protective efficacy was also studied by using the mean number of days which the mice survived after being given different challenge doses (Fig. 3). The survival against challenge with *S. dublin* SVA47 was almost the same in mice vaccinated with *S. dublin* SL5631 as with the hybrid *S. dublin* SL7103. Only with the two highest challenge doses of SVA47 (10^6 and 10^7 CFU) was a poorer protective efficacy of SL7103 than that of SL5631 seen (Table 2; Fig. 3A).

The hybrid SL7103 vaccine also protected against *S. typhimurium* challenge (Table 2; Fig. 3B). However, the protection elicited by the SL7103 vaccine was less solid than that elicited by the *S. typhimurium* SL1479 vaccine. Even at the low dose of 10^3 CFU of SVA44, three of the mice vaccinated with SL7103 died (not shown).

Vaccination with any of the vaccine strains (SL1479, SL5631, or SL7103), although protective of the mice, failed to eradicate either of the virulent challenge strains SVA44 and SVA47. Even with low challenge doses, the challenge strain could be recovered in some mice at autopsy 30 days after challenge (not shown). All mice were apparently healthy when sacrificed. The spleens were slightly enlarged and showed lymphatic hyperplasia and activated germinal centers. At low challenge doses, no gross pathological changes were seen. However, at high challenge doses ($\geq 10^5$ CFU) and in particular with the *S. dublin* SVA47 wild-type strain, macroscopic purulent foci could be seen in the liver, and the spleen was enlarged and showed lymphatic hyperplasia with activated germinal centers.

Anti-LPS antibody responses after *S. dublin* SL5631 vaccination. The enzyme immunoassays were done on sera pooled from each group of 10 mice. Mice intraperitoneally vaccinated with *S. dublin* SL5631 *aroA* live vaccine responded with IgM antibody titers against *S. dublin* SVA47 (aro^+, virulent) LPS (Fig. 4A). Increases were seen after two vaccinations and, in particular, at 2 weeks after the third immunization. The IgG response appeared later but was

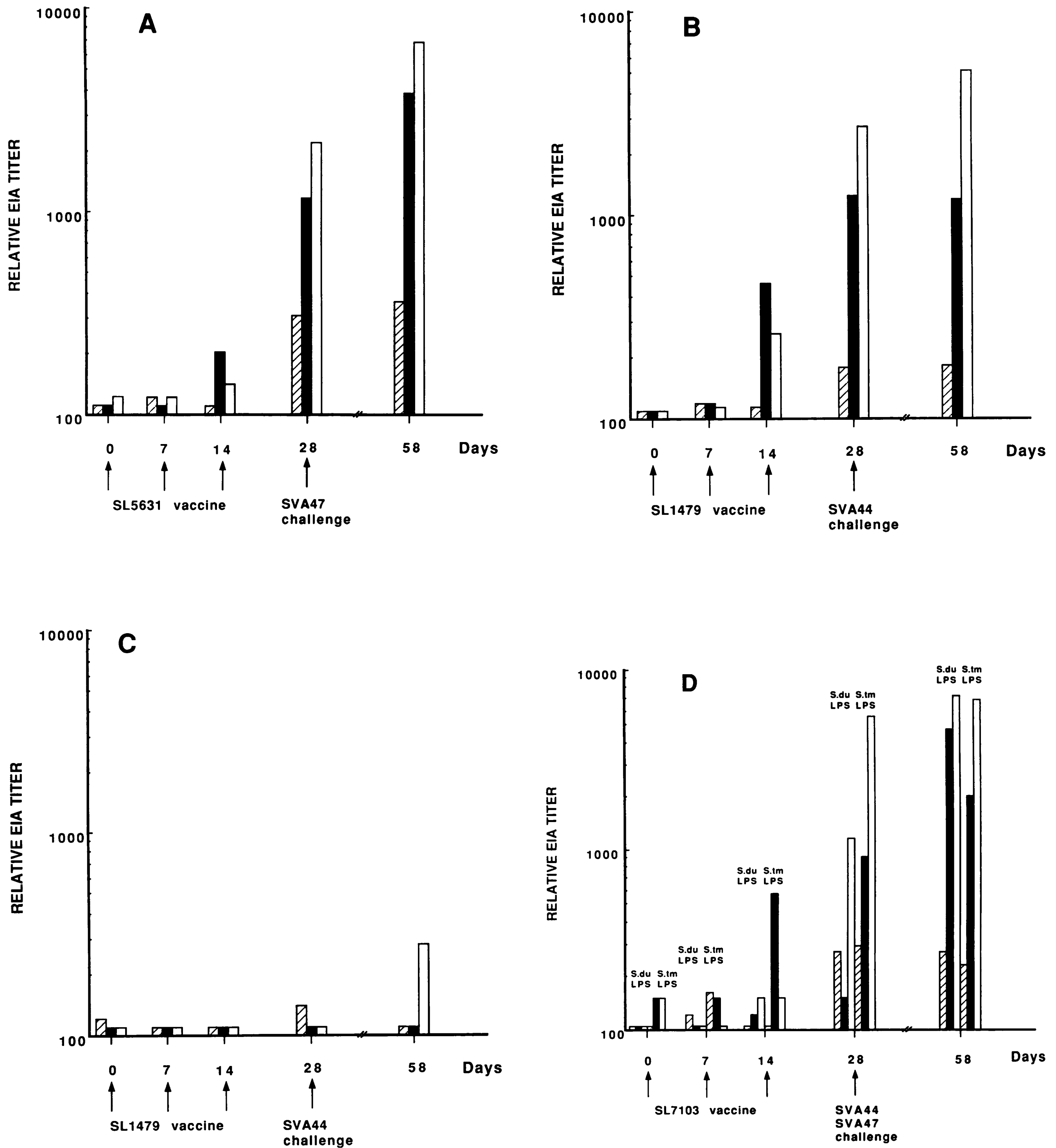

FIG. 4. Serum IgA, IgM, and IgG EIA antibody titers in vaccinated NMRI mice. Symbols: hatched bar, IgA; solid bar, IgM; open bar, IgG. (A) Mice intraperitoneally vaccinated with ca. 2×10^5 live *S. dublin* SL5631 bacteria on days 0 (prebleed), 7, and 14. The mice were challenged with 2×10^5 live *S. dublin* SVA47 bacteria. *S. dublin* LPS was used as the antigen. (B) Mice intraperitoneally vaccinated with ca. 2×10^5 live *S. typhimurium* SL1479 bacteria on days 0, 7, and 14. The mice were challenged with 10^6 live *S. typhimurium* SVA44 bacteria. *S. typhimurium* LPS was used as the antigen. (C) Sera from the same mice as those described for panel B but with *S. thompson* LPS as the antigen. (D) Mice intraperitoneally vaccinated with ca. 2×10^5 live *S. dublin* O4-O9 SL7103 bacteria on days 0 (prebleed), 7, and 14. The mice were challenged with either *S. dublin* SVA47 (S. du) or *S. typhimurium* SVA44 (S. tm) on day 28. Day 58 values show *S. dublin* LPS titers after *S. dublin* SVA47 challenge and *S. typhimurium* LPS titers after *S. typhimurium* SVA44 challenge.

much higher 2 weeks after the final dose (day 28). The IgA responses were relatively weak, even after three vaccinations. No titer increases against the *S. thompson* IS40 (O6,7) LPS antigen used as a control were seen (not shown).

Mice immunized with *S. dublin* SL5631 and surviving the challenge with 2×10^5 CFU of wild-type *S. dublin* SVA47 (7 of 10 mice) were sacrificed on day 58, and the sera were pooled and assayed. The IgA, IgM, and IgG titers had all increased about threefold as a result of the challenge infection (Fig. 4A). Also, an *S. thompson* LPS IgG titer of 360, which was 25-fold lower than that against the *S. dublin* LPS, was seen.

Anti-LPS antibody responses after *S. typhimurium* SL1479 vaccination. Mice immunized intraperitoneally with *S. typhimurium* SL1479 (2×10^5 live bacteria on days 0, 7, and 14) responded with anti-*S. typhimurium* SH4809 LPS IgM titers which, 2 weeks after the third vaccination, were 15-fold higher than those in sera collected before immunization (Fig. 4B). The IgG response appeared somewhat later but, after three vaccine doses, was more than 40-fold higher than that before vaccination. The IgA antibody response was weak. No titer increases against the *S. thompson* LPS antigen used as a control were seen (Fig. 4C).

Mice surviving the challenge with 10^6 live *S. typhimurium* SVA44 bacteria (8 of 10 mice) were sacrificed on day 58, and sera were pooled and assayed. The titers against the *S. typhimurium* LPS were the same as those before challenge for IgA and IgM and had almost doubled for IgG (Fig. 4B). Against the *S. thompson* LPS antigen, an IgG titer increase was seen (Fig. 4C) which was 30-fold lower than that against the *S. typhimurium* LPS antigen.

Anti-LPS antibody responses after vaccination with the *S. dublin* hybrid SL7103. The intraperitoneal vaccination with the hybrid strain SL7103 on days 0, 7, and 14 with 2×10^5 live bacteria resulted in anti-LPS antibody responses (Fig. 4D). The anti-*S. dublin* LPS response was not seen until 2 weeks after the third and final dose (day 28) and then predominantly in the IgG subclass. The IgM response was distinctly lower, but the IgG response was only half of the response seen after vaccination with the *S. dublin* SL5631 host strain (Fig. 4A). One month after challenge, the *S. dublin* LPS titers were equal to those seen in *S. dublin* SL5631-vaccinated and challenged mice (Fig. 4A and D).

An anti-*S. typhimurium* LPS response in IgM was already evident in the SL7103-vaccinated mice after two vaccinations (day 14), and a pronounced response was evident in all immunoglobulin classes tested 2 weeks after the final vaccination (day 28) (Fig. 4D). In serum samples taken 1 month after SVA44 challenge, the anti-*S. typhimurium* LPS titers were equal to those in mice vaccinated with *S. typhimurium* SL1479 and challenged with SVA44.

There are structural similarities between the O-antigenic polysaccharides in the LPSs from *S. dublin* and *S. typhimurium*, which share the same →2α-D-mannose-1→4α-L-rhamnose-1→3α-D-galactose-1→trisaccharide and only differ in that tyvelose in *S. dublin* and abequose in *S. typhimurium* are α1,3-linked to D-mannose in the repeating units. Therefore, the titers given in Fig. 4D represent specific as well as cross-reactive antibody responses. The pooled mouse sera were therefore absorbed (with *S. typhimurium* bacteria for anti-*S. dublin* LPS titer determinations and vice versa) before the EIA. Because of the shortage of mouse serum, an anti-mouse immunoglobulin conjugate estimating IgA, IgM, and IgG responses had to be used. After absorptions, it was evident that vaccination with the SL7103 hybrid resulted predominantly in an *S. typhimurium*, i.e., anti-O4, antibody response (Fig. 5).

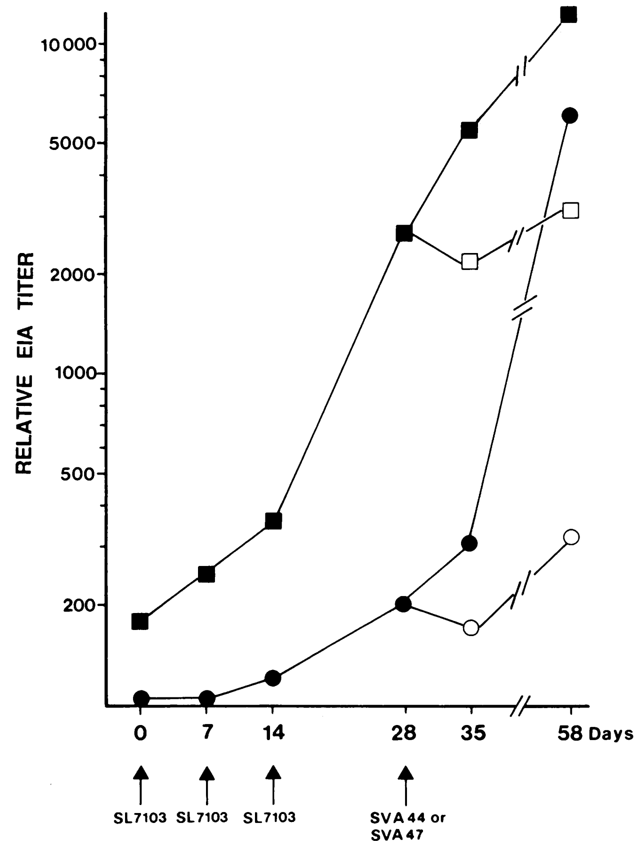

FIG. 5. Anti-*S. typhimurium* and *S. dublin* LPS titers in NMRI mice intraperitoneally vaccinated with the live *S. dublin* O4-O9 hybrid strain SL7103 and subsequently challenged intraperitoneally with either *S. dublin* SVA47 or *S. typhimurium* SVA44. Pooled sera were absorbed with heat-inactivated *S. typhimurium* prior to *S. dublin* LPS titer assays and with heat-inactivated *S. dublin* prior to *S. typhimurium* LPS titer assays. The titers were estimated in an EIA with sera diluted 1:1,000 and a sheep anti-mouse immunoglobulin conjugate. Symbols: ■, *S. typhimurium* LPS titers; □, *S. typhimurium* LPS titers after *S. dublin* SVA47 challenge; ●, *S. dublin* LPS titers; ○, *S. dublin* LPS titers after *S. typhimurium* SVA44 challenge.

Subsequent to challenge with *S. dublin* SVA47 of mice vaccinated with the SL7103 hybrid strain, the anti-*S. dublin* LPS titers, i.e., anti-O9, increased and were as high as those in mice vaccinated with *S. dublin* SL5631 and subsequently challenged with *S. dublin* SVA47 (Fig. 4A and 5). The *S. typhimurium* SVA44 challenge gave only a marginal increase in anti-*S. dublin* LPS antibody titers. Likewise, challenge with *S. typhimurium* SVA44 resulted in a significant increase in anti-*S. typhimurium* LPS titers, whereas the *S. dublin* SVA47 challenge did not cause any changes in *S. typhimurium* LPS titers (Fig. 5).

IgA, IgM, and IgG antibody titers against the *S. thompson* LPS used as a control antigen remained low, i.e., <150, after vaccination with SL7103. Subsequent to challenge with *S. dublin* SVA47 or *S. typhimurium* SVA44 on day 28, the *S. thompson* LPS IgG titers on day 58 were 1,080 and 860, respectively. No IgA or IgM relative titers of >200 were seen after either challenge.

DISCUSSION

We utilized the experimental mouse model (12) to study the protective effect of intraperitoneal vaccination with a live auxotrophic hybrid strain, SL7103, which is *S. dublin* SL5631 (O9,12) with the O-antigen-specifying chromosomal *rfb* locus from *S. typhimurium* (O4,12) added to its gene complement. Strain SL7103 thus expressed both the original host O9 epitope (specified by the α-tyvelose-1→3α-D-mannose-1→disaccharide) and the foreign O4 epitope (specified by the α-abequose-1→3α-D-mannose-1→disaccharide) as seen in indirect immunofluorescence studies with the O4 and O9 epitope-specific rabbit antisera raised with the synthetic disaccharide-bovine serum albumin conjugates and with mouse monoclonal O4-specific and O9-specific antibodies (4, 17, 35). Qualitative and quantitative sugar analyses also showed that abequose and tyvelose were present in a ratio of approximately 1:1. Although not studied by structural analysis in this communication, we recently showed that two other hybrid constructs, each with *rfb* loci from both *S. typhimurium* (O4,12) and *S. enteritidis* (O9,12), also likewise expressed the O4 and O9 epitopes in about equal ratios and that an O chain in the LPS of these strains could be composed of repeating tetrasaccharide units of the two different kinds (35). There is no reason to assume that the O-antigenic polysaccharide chains of strain SL7103 should differ from the two recently studied.

NMRI mice given a single dose of either 2×10^5 or 2×10^7 CFU intraperitoneally cleared the bacteria from the intestines and livers within 21 days and from the spleens within 28 days (Fig. 2). When mice were given 2×10^5 CFU once a week in three doses, all organs were clear of bacteria on day 28 (Fig. 2). The clearance of the vaccine strains from NMRI mice appears to be more rapid than that of similar aromatic-dependent strains from BALB/c mice (21). Since the protection afforded by a single vaccine dose was negligible compared with that from a regimen of three doses, subsequent studies were done in mice given three doses and challenged 2 weeks later (day 28), when nonspecific immunity should play only a marginal role.

NMRI mice vaccinated three times and challenged with either virulent *S. dublin* SVA47 or virulent *S. typhimurium* SVA44 2 weeks later were protected against both challenge infections, with LD_{50}s of 1.6×10^4 and 1.0×10^5 CFU, respectively (Table 2). Compared with the protection elicited by either auxotrophic *aroA* vaccine (*S. dublin* SL5631 or *S. typhimurium* SL1479), the protection elicited by the hybrid SL7103 (which is *aroA recA*) was approximately 70-fold lower for the *S. dublin* challenge and 100-fold lower for the *S. typhimurium* challenge (Table 2; Fig. 3).

NMRI mice vaccinated intraperitoneally with SL7103 responded with higher antibody titers against the *S. typhimurium* O antigen than against the *S. dublin* O antigen. This was evident when either unabsorbed or absorbed pooled sera were used in the EIAs (Fig. 4D and 5). Mice immunized with either live *S. dublin* (Fig. 4A) or live *S. typhimurium* (Fig. 4B) responded at about the same time and with responses of quite similar titers. This suggests that in immunization with the hybrid SL7103 strain, the anti-*S. typhimurium* LPS response was favored over the anti-*S. dublin* LPS response. At which level this discrimination is operative is unknown. In spite of the high anti-*S. typhimurium* LPS antibody response, the protective effect elicited by the hybrid vaccine was about 100-fold lower than that seen after vaccination with *S. typhimurium* SL1479 (Table 2). On the other hand, it was 10-fold better than the protection elicited by the *aroA* mutant SL7368 originating from SVA44. In a similar way, the protection elicited by the hybrid vaccine against the *S. dublin* challenge was about 70-fold lower than that elicited by *S. dublin* SL5631. This suggests that besides humoral immunity directed against the O antigen, either humoral immunity against other bacterial structures or other types of immune responses are operative in the protection seen. The data also indicate that the introduction of the *S. typhimurium rfb* gene cluster and the *recA* characteristic may have impaired the protective effect of the *S. dublin* hybrid vaccine against *S. dublin* challenge.

NMRI mice vaccinated with the live *S. dublin* SL5631 strain were protected against challenge with wild-type *S. dublin* SVA47 (Table 2). The vaccination increased the LD_{50} 100,000-fold in comparison with that of nonimmunized mice. However, no, or only marginal, protection was seen against challenge with *S. typhimurium* SVA44. Likewise, vaccination with live *S. typhimurium* SL1479 increased the LD_{50} for challenge with *S. typhimurium* SVA44 about 1,000,000-fold compared with that of nonvaccinated mice and gave almost no protection against *S. dublin* SVA47 (Table 2). This results in the conclusion that, on the day of challenge (day 28), nonspecific host defense mechanisms should have played only a marginal role. Cultured livers and spleens on day 28 were also clear of the vaccine strains.

The demonstration of the O-antigen-specific protection merely confirms earlier studies (2, 5, 8, 17–19, 23, 24). The O-antigen specificity of the protection was a consequence of elicited antibodies specific for the O4 and O9 epitopes in mice vaccinated with *S. typhimurium* SL1479 and *S. dublin* SL5631, respectively. The fact that both strains share the O12 epitope, the determinant(s) of which is found in the common 1→α-D-mannose-1→4α-L-rhamnose-1→3α-D-galactose-1→trisaccharide, was apparently not enough to elicit a demonstrable cross-protection. We could not specifically estimate the anti-O12 titers, but the cross-reactivity in the EIA suggested that the O4 and O9 titers were at least 5- to 10-fold higher than the O12 titers (data not shown). The immune response seen after three doses was predominantly that of IgG, followed by IgM and IgA (Fig. 4A and B). Mäkelä and coworkers showed that only IgM antibodies protected against experimental mouse salmonellosis (27, 28). We demonstrated in studies of the protective effect of mouse monoclonal antibodies, specific for O-antigen epitopes and passively administered to NMRI mice, that both IgG and IgM antibodies which were O9 specific were protective but that only the IgM subclass of O12-specific antibodies provided significant protection (4). One possible explanation of the observed poor cross-protective effect in this study after *S. dublin* vaccination and *S. typhimurium* challenge and vice versa is because few, or no, anti-O12-specific antibodies were generated after vaccination with either *S. typhimurium* SL1479 or *S. dublin* SL5631.

We do not know why the hybrid (O4-O9) live vaccine caused a higher O4-specific than O9-specific antibody response (Fig. 5). It may reflect an inherently higher immunogenic activity of the abequose-containing polysaccharide than of the tyvelose-containing polysaccharide. The EIA titers in pooled sera of mice given the O4 live vaccine were not consistently higher than those of the pooled sera from mice given the O9 live vaccine (Fig. 4A and B).

In this investigation, both the anti-LPS antibody response and the protective efficacy of each of the two monospecific, O4 or O9, live vaccines was almost entirely O specific. The same was true in the investigation of Lyman et al. (18), in which protection was measured by counts of viable chal-

lenge bacteria of each type in the livers and spleens of mice immunized with several doses of killed bacteria. In some other systems, however, animals immunized by the administration of an O4 or O9 strain, live or killed, have a degree of nonspecific immunity when tested within a month or so of the last vaccine dose (8, 30). The reasons for these variable results are not apparent.

The ability of live vaccines to reduce deaths from *Salmonella* challenge has been seen in many investigations, but the mechanism(s) of protection is not known in detail (5, 21, 33). The O specificity of protection seen in our experiments which we attribute to O antibodies failed, however, to eradicate the challenge bacteria.

In conclusion, the stable *S. dublin* SL7103 hybrid strain, expressing equal amounts of the O4 and O9 epitopes, elicits protective immunity against challenge with either a virulent *S. typhimurium* or a virulent *S. dublin* (Table 2). This confirms the important role of LPS in the pathogenesis of salmonellosis (19) and convincingly demonstrates that an anti-LPS antibody response effectively protects against an intraperitoneal challenge in the experimental mouse typhoid model. The protective efficacy of the hybrid O4-O9 strain was, however, about 100-fold less (but in one instance 10-fold better) than that of the parental *S. dublin* and *S. typhimurium* strains.

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