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Salmonella typhimurium Infection in Calves: Cell-Mediated and Humoral Immune Reactions Before and After Challenge with Live Virulent Bacteria in Calves Given Live or Inactivated Vaccines

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Groups of six calves, 4 to 5 weeks old, were vaccinated either orally with a live auxotrophic *Salmonella typhimurium* (O-antigen 1,4,12) SL1479 vaccine (10^8 bacteria on day zero, 10^{10} bacteria on days 7 and 14) or subcutaneously with a heat-inactivated (56°C, 30 min) *S. typhimurium* SVA1232 vaccine (10^{10} bacteria suspended in 30% [vol/vol] aluminum hydroxide on days zero, 7, and 14). The calves were then orally challenged with either 10^6 (~100× the 25% lethal dose) or 10^9 (~100,000× the 25% lethal dose) live bacteria of the calf-virulent *S. typhimurium* SVA44 strain. The immune reactivity of these calves and of nonvaccinated control calves was followed before and after the challenge infection up to 42 days by (i) intradermal injection of *S. typhimurium* crude extract, outer membrane protein preparation (porins), and lipopolysaccharide (LPS), (ii) in vitro stimulation of peripheral blood lymphocytes estimated by using uptake of [^{3}H]thymidine, with *S. typhimurium* crude extract, porins, LPS, and polysaccharide (O-antigenic polysaccharide chain free of lipid A), and *Salmonella* sp. serotype *thompson* (O-antigen 6,7) strain IS40 LPS and polysaccharide, and (iii) estimation of the class-specific immunoglobulin G (IgG) and IgM antibody responses against *S. typhimurium* LPS and porins, and *Salmonella* sp. serotype *thompson* LPS. The immune studies showed that in calves given the live vaccine orally, the skin test reactivity and lymphocyte stimulation indices were significantly higher (P values ranging from <0.025 to <0.0005) against homologous, but not heterologous, antigens than those seen in calves given the heat-inactivated vaccine subcutaneously. In contrast, the IgG and IgM antibody titers against homologous LPS and porins were significantly higher ($P < 0.0005$) in sera collected on day 21 from calves given the heat-inactivated vaccine than in calves given the live vaccine. After the oral challenge, calves given the live vaccine showed reduced cell-mediated immune reactions, in agreement with the observation that the host defense could eradicate the challenge organism, whereas calves given the heat-inactivated vaccine showed significantly increased cell-mediated immune reactions (P values ranging from <0.025 to <0.005), in agreement with the observation that in these calves, the challenge strain caused enteritis as well as systemic invasion. The increased cell-mediated immune reactivity in calves given the live vaccine correlated well with the excellent protection against challenge infection seen in these animals.

Salmonella infections result in stimulation of both humoral and cell-mediated immunity (5, 22, 24). The specificity of these immune reactions is directed against components of the envelope of the *Salmonella* bacteria, like the O-antigenic polysaccharide (PS) chain of the lipopolysaccharide (LPS) and envelope proteins such as porins (22, 24). Although the humoral immunity contributes to the host defense against *Salmonella* bacteria, the ability of the components of the cellular immune systems to eradicate the invading organism has been considered to be of overriding importance (12).

Salmonella infections in cattle are of worldwide public health concern; therefore, numerous attempts have been made to protect the cattle by immunization (1, 3, 4, 19, 20, 25, 27). Limited protection has resulted in some instances, and the experiences seem to favor the use of live rather than killed vaccines. This opinion is also

in agreement with the results obtained in immunization studies with experimental animals (5, 10) and with the observation that a live bacterial vaccine, like a natural infection, elicits cell-mediated immune responses to a much greater extent than does a killed vaccine (5).

In the accompanying paper (23), we immunized calves with either a live nonvirulent *Salmonella typhimurium* vaccine given orally or a heat-inactivated and killed *S. typhimurium* vaccine given subcutaneously, and we studied the protection against oral challenge with the calf-virulent *S. typhimurium* SVA44 strain. In this communication, we study the immune responses, cellular and humoral, after vaccination and after the challenge infection. The cellular immune responses were estimated both by intradermal injection of various antigens from the *Salmonella* cell envelope (LPSs and porins) and by following the [^{3}H]thymidine uptake in peripheral blood lymphocytes (PBLs) after stimulation with the same antigens. The humoral antibody response was estimated by using enzyme-linked immunosorbent assay (ELISA) with LPSs and porins as antigens.

MATERIALS AND METHODS

Experimental animals. The experimental calves, 4 to 5 weeks old and of both sexes, and the numbering of the groups (I to V), were the same as in the accompanying paper (23).

Bacterial strains. The bacterial strains used in the experiment are listed in the accompanying paper (23).

Antigen preparations. *S. typhimurium* SVA44 (O 4,5,12) crude extract, *S. typhimurium* SH4809 LPS (O 4,5,12), *S. typhimurium* SH4809 PS (O 4,12), *Salmonella* sp. serotype *thompson* IS40 (O 6,7), *Salmonella* sp. serotype *thompson* PS (O 6,7), and outer membrane proteins (porins) from *S. typhimurium* SL1909 were available from previous studies (22, 24).

Preparation of immunogens and immunization procedures were as described in the accompanying paper (23).

Estimation of delayed skin reactions. The skin tests were carried out as previously described (24) on all calves on days zero, 21, and 42. The double skin fold thickness increases (in millimeters) were measured by using a slide calliper 48 h after intradermal injections of 0.2 ml of the various antigen preparations.

Isolation of lymphocytes. Blood lymphocytes were collected and isolated by the Ficoll-Isopaque method as described previously (22). Lymphocyte stimulation tests were performed as described previously (22). Each antigen was tested in four wells of Microplates (Sterlin Ltd., Teddington, England), and the controls with phytohemagglutination and without any mitogen were tested in sex wells each. The highest and the lowest values of counts per minute (cpm) for each antigen and for control groups were discarded. The stimulation index (SI) was calculated according to the formula SI = (mean cpm of the two wells stimulated with antigen)/(mean cpm of four control wells). All lymphocytes responded to stimulus with phytohemagglutination (Pharmacia, Uppsala, Sweden); SI values were >5. The [^{3}H]thymidine uptake in PBLs which had not been stimulated with any antigen varied from 730 to 2,140 cpm, with a mean value of 1,730 (standard deviation, 650 cpm).

ELISA. The ELISA was performed as described earlier (24). Alkaline phosphatase (calf intestinal mucosa type VII; Sigma Chemical Co., St. Louis, Mo.) conjugated to rabbit anti-bovine immunoglobulin M (IgM) and IgG (Miles Biochemicals, Stoke Poges, Slough, England) was used, and the absorbance was measured at 405 nm in a Titertek Multiscan Plate Reader (made for Flow Laboratories by Eflab, Oy, Helsinki, Finland). All ELISA titers are expressed as endpoint titers, i.e., the reciprocal of the serum dilution yielding a change of 0.1 in an optical density of 405 nm in 100 min.

Statistical analyses. Student's t test and a χ^2 test were used for statistical analyses as in the accompanying paper (23).

RESULTS

The immunization and challenge schemes of the five groups of calves are given in Table 1 of reference 24. The immune reactivity of these calves was studied throughout the vaccination and challenge period by (i) meaurement of the skin fold thickening, e.g., delayed hypersensitivity-like skin reactivity, after subcutaneous injection of a crude extract from *S. typhimurium* SVA44, a purified outer membrane protein preparation (34,000- and 36,000-dalton porins) from *S. typhimurium* SL1907, and a purified LPS from *S. typhimurium* SH4809, (ii) uptake of [^{3}H]thymidine by PBLs stimulated by *S. typhimurium* SVA44 crude extract, *S. typhimurium* SL1907 porin preparation, *S. typhimurium* SH4809 LPS, and PS (LPS rendered free of lipid A by acid hydrolysis), *Salmonella* serotype sp. *thompson* strains IS40 LPS and PS, and (iii) humoral IgG and IgM antibody response against three antigens: *S. typhimurium* SH4809 LPS, *S. typhimurium* SL1907 porins, and *S. thompson* IS40 LPS as measured in ELISA.

Skin reactivity. Nonimmunized calves (group I) were skin tested on days zero and 21. In all instances, the skin swelling was <2.0 mm (mean, 1.0 mm). No increase was seen from day zero to day 21. Calves vaccinated subcutaneously with the heat-inactivated *S. typhimurium* SVA1232 vaccine (groups II and III) responded with significant increases in skin reactivity against the three antigens used when tested on day 21: crude extract, porin preparation, and LPS (Fig. 1; $P < 0.01$, $P < 0.005$, and $P < 0.0025$, respectively, versus test results on day zero). In calves given the attenuated live *S. typhimurium* SL1479 vaccine orally (groups IV and V), the skin reactivities seen on day 21 against all three antigens, and in particular the crude extract and porin antigens, were much more pronounced ($P < 0.0005$ versus day zero

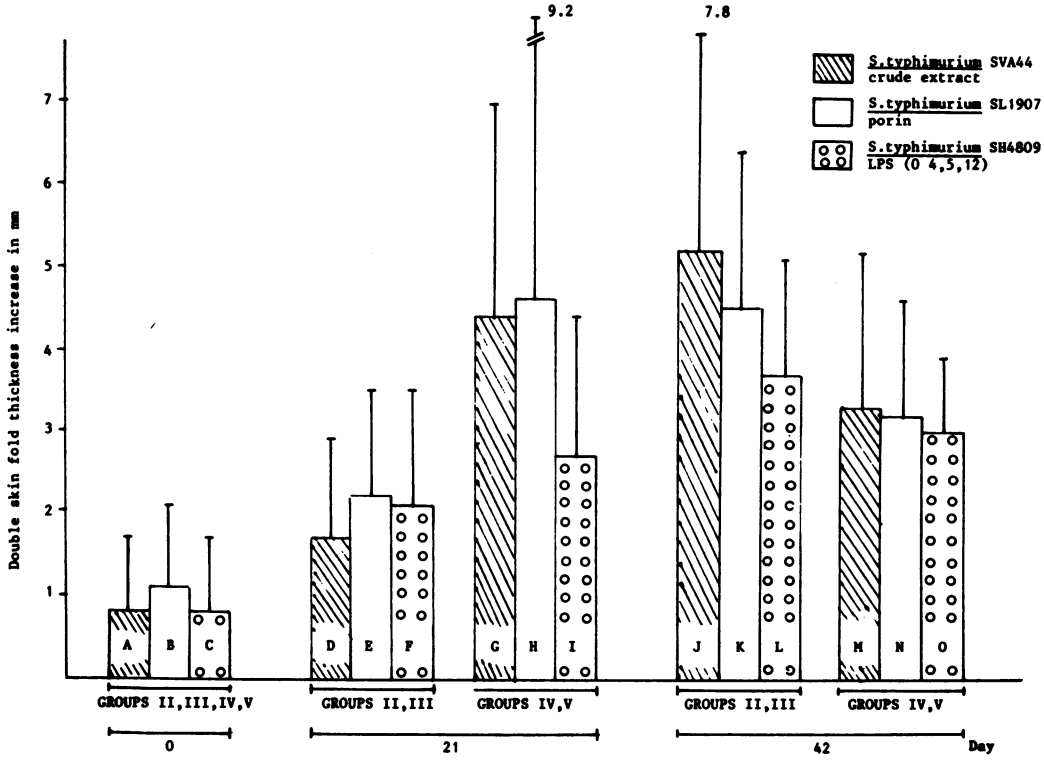

FIG. 1. Double skin fold thickness increases in calves before vaccination (day zero), after vaccination with *S. typhimurium* SVA1232 (O 4,5,12) inactivated whole cell vaccine (groups II and III) and live *S. typhimurium* SL1479 (O 1,4,12) (groups IV and V; day 21), and after challenge infection with *S. typhimurium* SVA44 (O 4,5,12; day 42). Each bar shows the mean double skin fold thickness increase in millimeters and the standard deviation. Statistical analyses were made by using Student's *t* test (23). Level of statistical significances: A versus D, $0.005 < P < 0.01$; B versus E, $0.025 < P < 0.005$; C versus F, $0.001 < P < 0.0025$; A versus G, $P < 0.0005$; B versus H, $P < 0.0005$; C versus I, $P < 0.0005$; A versus J, $P < 0.0005$; B versus K, $P < 0.0005$; C versus L, $P < 0.0005$; A versus M, $P < 0.0005$; B versus N, $P < 0.0005$; C versus O, $P < 0.0005$; D versus J, $P < 0.0005$; E versus K, $0.0025 < P < 0.005$; F versus L, $0.025 < P < 0.05$; G versus M, $P > 0.1$; H versus N, $P > 0.1$; I versus O, $P > 0.1$; D versus G, $0.001 < P < 0.0025$; E versus H, $0.025 < P < 0.05$; F versus I, $P > 0.1$; J versus M, $0.025 < P < 0.005$; K versus N, $0.05 < P < 0.1$; L versus O, $P > 0.1$.

testing for all three antigens, and $P < 0.025$, $P < 0.05$, and $P > 0.25$, respectively, versus day 21 testing in the calves given heat-inactivated vaccine). When calves were tested on day 42 (21 days after oral challenge with the virulent *S. typhimurium* SVA44 strain), the findings were quite different (Fig. 1): calves in the two groups (II and III) given the heat-inactivated vaccine showed larger skin swellings after injection of the test antigens ($P < 0.0005$ versus day zero testing; $P < 0.0025$ versus day 21 testing). In contrast, calves vaccinated with the oral live vaccine showed smaller skin swellings on day 42 than on day 21 ($P > 0.25$).

Stimulation of PBLs. The ability to stimulate PBLs was followed in three of the groups of calves: group I (nonvaccinated), group II (given heat-inactivated vaccine and challenged with 100× the 25% lethal dose [LD_{25}]), and group IV (given the live vaccine orally and challenged with 100× the LD_{25} [Table 1]). Tests were done on PBLs collected on days zero, 7, 21, 28, and 42.

Calves in the control group I, as expected, had lymphocytes which were not stimulated by any of the six antigens used. It was evident, however, that after the challenge infection on day 21, a marked and specific increased uptake in the PBLs could be observed when they were stimulated with the *S. typhimurium* antigens on day 28 (Table 1). No statistical significance was seen, most likely because only two of six calves were still alive on day 28. Later testings could not be done in group I because on day 42 the remaining two calves had succumbed to the challenge infection.

PBLs from calves given the heat-inactivated vaccine (group II) responded with a significantly

TABLE 1. SIs measured as [^{3}H]thymidine uptake in PBLs from nonvaccinated calves (group I), calves vaccinated with *S. typhimurium* SVA1232 (O 4,5,12) inactivated whole cell vaccine (group II), and calves vaccinated with live *S. typhimurium* SL1479 (O 1,4,12) (group IV)[a]

Antigen used for stimulation	Mean $\log_{10}$ SI (±SD) in PBLs collected on day[b]:				
	Zero	7	21	28	42
Group I					
S. typhimurium					
Crude extract	0.05 (0.10)		0.07 (0.15)	0.22 (0.06)	
Porin	0.04 (0.07)		−0.03 (0.24)	0.24 (0.09)	
LPS	0.12 (0.11)		0.03 (0.10)	0.24 (0.02)	
PS	0.07 (0.05)		0.04 (0.19)	0.37 (0.19)	
S. thompson					
LPS	−0.01 (0.16)		−0.02 (0.19)	0.25 (0.07)	
PS	−0.12 (0.06)			−0.08 (0.11)	
Group II					
S. typhimurium					
Crude extract	0.03 (0.25)	0.15 (0.43)	0.13 (0.13)	0.50*** (0.31)	0.29* (0.11)
Porin	−0.01 (0.12)	0.29*** (0.11)	0.21** (0.14)	0.31*** (0.09)	0.32*** (0.09)
LPS	−0.14 (0.10)	0.26 (0.18)	0.16 (0.20)	0.36** (0.16)	0.26* (0.06)
PS	−0.02 (0.19)	0.21 (0.29)	0.11 (0.21)	0.29* (0.20)	0.25* (0.19)
S. thompson					
LPS	0.10 (0.25)	−0.04 (0.30)	0.09 (0.16)	−0.05 (0.11)	0.06 (0.07)
PS	0.04 (0.12)	0.19* (0.15)	0.02 (0.11)	−0.13 (0.16)	0.02 (0.09)
Group IV					
S. typhimurium					
Crude extract	−0.13 (0.22)	0.30** (0.26)	0.52** (0.31)	0.35** (0.26)	0.30** (0.26)
Porin	−0.11 (0.24)	0.24* (0.28)	0.40** (0.10)	0.30* (0.30)	0.18 (0.40)
LPS	−0.11 (0.25)	0.25* (0.15)	0.17* (0.17)	0.13 (0.10)	0.27 (0.13)
PS	−0.06 (0.21)	−0.09 (0.18)	−0.01 (0.16)	0.27* (0.15)	−0.12 (0.14)
S. thompson					
LPS	0.07 (0.12)	−0.13 (0.39)	0.02 (0.28)	0.17 (0.23)	0.04 (0.09)
PS	−0.05 (0.07)	−0.20 (0.32)	−0.02 (0.19)	−0.07 (0.11)	−0.10 (0.21)

[a] Calves were vaccinated on days zero, 7, and 14 and were challenged with 100× the LD_{25} of *S. typhimurium* SVA44 on day 21.

[b] Statistical significance versus the SI at day zero; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

increased ability to take up the [^{3}H]thymidine, particularly when they were stimulated with the porin preparation (days 7 and 21). Although increases were also noted with the other *S. typhimurium* antigens, the differences were not statistically significant. After the *S. typhimurium* SVA44 challenge infection on day 21, significant increases with all four *S. typhimurium* antigens were observed in all instances when tested on days 28 and 42, whereas no increases were observed with the two heterologous *Salmonella* serotype sp. *thompson* antigens, which served as controls, in any of the instances tested. The high SI values noted for the porin preparation on days 7 and 21 remained at the same level after the challenge infection.

The PBLs from calves given the live vaccine orally (group IV) responded with significantly increased uptakes of [^{3}H]thymidine when they were stimulated with the *S. typhimurium* crude extract, porin preparation, and LPS in five of the six instances when tested on days 7 and 21. After the challenge infection, the SI values were lower for the *S. typhimurium* crude extract, porin preparation, and LPS in six of six instances on days 28 and 42, as compared with the results on day 21. In no instance was any increased uptake observed when the PBLs were stimulated with the heterologous *Salmonella* serotype sp. *thompson* antigens.

Humoral antibody response. The IgM and IgG titers in serum samples from the calves against *S. typhimurium* SH4809 LPS (O-antigens 4,5,12), SL1907 porins, and *Salmonella* sp. serotype *thompson* IS40 LPS (O 6,7) were estimated by using ELISA. On day zero, serum samples from all calves had $\log_{10}$ endpoint titers <3.0 (Fig. 2). After subcutaneous vaccination with a heat-inactivated *S. typhimurium* SVA1232 vaccine, the calves in groups II and III responded with significantly increased anti-LPS and anti-porin IgM and IgG titers on day 21 ($P < 0.0005$). In calves given the live *S. typhimurium* SL1479 vaccine by mouth (groups IV and V), the porin IgG and IgM titers were approximately equal to those seen in calves in groups II and III, whereas the anti-LPS IgM and IgG titers were more than 10-fold lower ($P < 0.0005$). In serum sam-

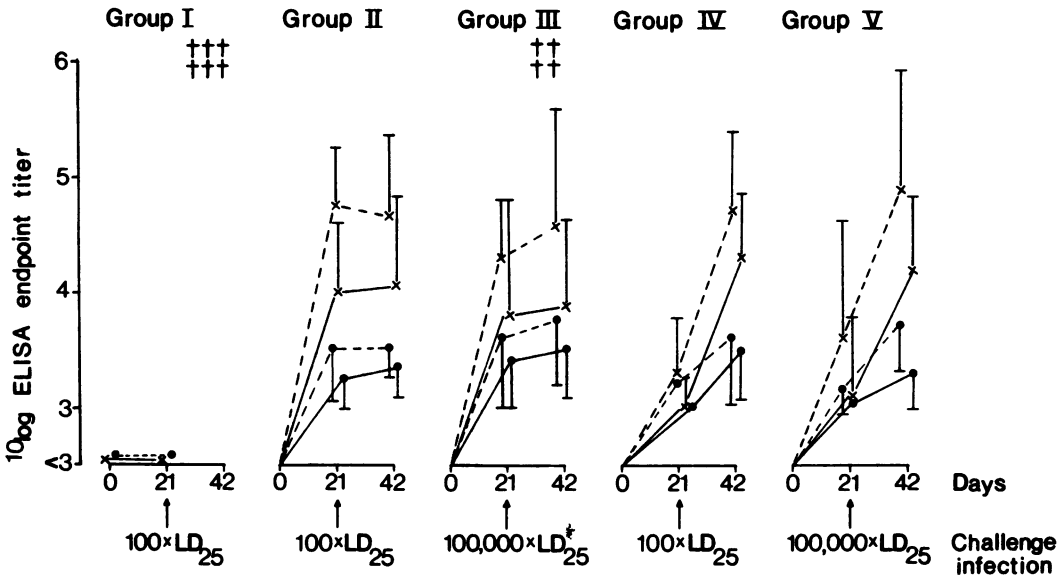

FIG. 2. Class-specific IgM and IgG antibody titers in calves after vaccination and challenge with *S. typhimurium*. The vaccination procedures, challenge infections, and group designations are as in Fig. 1. Titers are expressed as $\log_{10}$ endpoint ELISA titers (mean and standard deviation). Symbols: *S. typhimurium* SH4809 LPS IgM (X--X) and IgG (X——X) titers; *S. typhimurium* SL 1907 porin IgM (●--●) and IgG (●——●) titers.

ples collected on day 42, the anti-LPS and anti-porin titers in calves in all four groups were almost equal ($P > 0.25$). When assays were done with the heterologous *Salmonella* sp. serotype *thompson* LPS antigen, all serum samples on all occasions had IgM and IgG $\log_{10}$ endpoint titers <3.0.

DISCUSSION

As a result of an infection in calves with either *S. typhimurium* or *Salmonella* sp. serotype *dublin*, cell-mediated as well as humoral immunity developed with specificity for outer membrane proteins and LPSs (22, 24). Because in a vaccination experiment, two oral sublethal doses of a living virulent *S. typhimurium* strain had been shown to afford protection superior to that of subcutaneous or oral vaccination with Formalin-killed bacteria (25), we found it interesting to analyze the cellular and humoral immune responses after two vaccination schemes: subcutaneous immunization with the heat-inactivated *S. typhimurium* SVA1232 strain and oral immunization with the live auxotrophic *S. typhimurium* SL1479 strain.

Using the development of a delayed skin swelling reaction after intradermal injection of a crude extract of *S. typhimurium* and purified and defined porins and LPSs, we found that the skin swellings were significantly larger for the crude extract and porin preparation in calves given the live vaccine orally (Fig. 1). It should be noted that the skin testing of control calves with the crude extract, porin, and LPS (50-μg doses of each) did not result in an increased reactivity between days zero and 21. Furthermore, three injections 10 days apart have likewise failed to increase the skin reactivity in unvaccinated calves (unpublished data). That the increase in skin swelling is specific with respect to the *S. typhimurium* LPS after a *S. typhimurium* infection has been demonstrated earlier (24); hence, heterologous LPS antigens were not used in this study. In the in vitro assays, using uptake of [^{3}H]thymidine in PBLs stimulated with four homologous (crude extract, porin, LPS, and PS) and the two heterologous (LPS and PS) antigens, the uptake in cells from calves vaccinated with the live vaccine was significantly higher with all of the homologous antigens tested than in PBLs from calves vaccinated with the heat-killed vaccine (Table 1). It should be noted that a significant uptake was seen in PBLs taken from calves vaccinated with the heat-inactivated vaccine both on day 7 and on day 21 when they were stimulated with the porin preparation (Table 1). This is not surprising since the heat inactivation of the vaccine took place at 56°C and porins are resistant to denaturation up to 70°C (2, 14). The fact that the live vaccine stimulated to a significant specific cell-mediated immune response against the crude extract, porins, and LPS, whereas the heat-inactivated vaccine stimulated

to a significantly increased uptake only when stimulated with the porins, is interesting with respect to the host defense, since multiplication and dissemination in the tissues of the challenge strain were prevented only in calves vaccinated orally with the live strain.

The humoral immune response as estimated in ELISA against the *S. typhimurium* LPS antigen developed more rapidly, and the titers were significantly higher, in calves given the heat-inactivated vaccine as compared with the calves given the live vaccine orally ($P < 0.0005$ for IgG and IgM [Fig. 2]). Because the calves with the higher humoral IgG and IgM antibody titers (groups II and III) were poorly protected, and we observed a poor ability of these calves to prevent penetration of the gastrointestinal mucosa and spread and multiplication of the challenge organism in their tissues (23), it is evident that the high humoral antibody titers correlated poorly to the host defense. This is in agreement with earlier experiences of *Salmonella* infections in mice (5, 8, 12) and humans (11) vaccinated with killed vaccines.

The course of development of the immune reactivities after the oral challenge infection was markedly different in calves that received the heat-inactivated vaccine when compared with those given the live vaccine. The skin reactivity against the crude extract and porins increased significantly ($P < 0.0025$ and $P < 0.05$, respectively) between days 21 and 42 in calves given the heat-inactivated vaccine (Fig. 1). Likewise, the [^{3}H]thymidine uptake of the PBLs when they were stimulated with either of the four antigens increased significantly on days 28 and 42 as compared with day 21 (Table 1). The increases were in all instances specific; no increased uptakes were noted when the unrelated *Salmonella* serotype sp. *thompson* LPS and PS antigens were used in the in vitro studies (Table 1). All results of the in vivo testing do indeed indicate that the calves given the heat-inactivated vaccine responded as nonvaccinated calves did to the *S. typhimurium* SVA44 infection (22, 24). On the other hand, in calves given the live vaccine strain orally (groups IV and V), skin reactivity on day 42 (Fig. 1) and PBL uptakes on days 28 and 42 (Table 1) decreased in comparison with the values seen on day 21. The time course observed is identical to what we saw after the sublethal *S. typhimurium* infection (22, 24) and suggests that the oral challenge infections with either 10^6 or 10^9 live *S. typhimurium* SVA44 bacteria did not stimulate the cell-mediated immune reactions. Supposedly, the host-defense mechanisms elicited by the live vaccine eliminated the challenge organisms, a hypothesis supported by the bacteriological examinations in the accompanying communication (23). Increases were seen in humoral antibody titers in these calves between days 21 and 42 (Fig. 2), but we do not take them as an indication of an antigenic stimulus by the challenge SVA44 strain, since in both time course and titer levels, they were similar to what was observed after the natural, sublethal infection (22, 24).

The fact that the calves vaccinated with the live vaccine orally, and with developed cell-mediated immunity against porins as well as LPS, were protected against an oral challenge dose of 10^9 live *S. typhimurium* SVA44 organisms (100,000× the LD_{25}) does not lead us to conclude that cell-mediated immune mechanisms are responsible for the observed and efficient host defense. The oral vaccination with the live *S. typhimurium* SL1479 strain most likely evoked IgA memory cells and T memory cells persisting at the site of antigen exposure, mostly in lymphoid follicles such as Peyer's patches (7, 21, 26). These memory cells mediate the secondary-type mucosal IgA response that follows challenge at the site of mucosal priming (13, 17, 18). That the immunizing bacteria have to be alive, since most likely they must penetrate into the lymphoid follicles, is strongly suggested by the repeated observations that oral vaccination with killed bacteria is ineffective (6, 8, 9).

Despite an efficient mucosal immunity, the possibility exists that some of the challenge *S. typhimurium* SVA44 bacteria penetrated through the gastrointestinal mucosa. The fact that calves challenged with either 100 or 100,000× the LD_{25} developed a moderate but significant pyrexia for 2 to 5 days (23) suggests that endotoxins were released into the circulation. The challenge bacteria, however, were eliminated from the tissues: only in a few instances was the SVA44 strain recovered from the tissues of these calves upon autopsy on day 42 (23). In mice, it appears that the *Salmonella* multiplication is controlled by a macrophage-dependent mechanism (15, 16). Although not studied in the calves so far, it is not unlikely that in them a similar mechanism is effective. It is tempting to speculate that the cellular immune mechanisms, to be effective, have to recognize not only proteins in the *Salmonella* cell envelope but also the O-antigen LPS. This is supported by the finding that only calves given the oral live vaccine developed significant immune reactivity against the LPS (Table 1). Experiences with live oral *Salmonella* vaccines in mice (8) and humans (9) have convincingly demonstrated that the presence of the O-antigenic PS chain is an important requisite in the vaccine to afford protection.

In conclusion, these vaccination studies have convincingly shown that the use of the live auxotrophic strain *S. typhimurium* SL1479 as an

oral vaccine evokes in calves an efficient protection against virulent *S. typhimurium* challenge. The exact mechanisms of this protection, i.e., the relative contributions of the local immunity on the mucosal level and cellular immunity in the tissues, remain to be elucidated. Furthermore, no studies have been performed on how an efficient vaccination schedule should be constructed for providing long-lasting protection under field conditions.

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