Salmonella typhimurium Infection in Calves: Specific Immune Reactivity Against O-Antigenic Polysaccharide Detectable in In Vitro Assays

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Peripheral blood lymphocytes collected from calves infected experimentally with Salmonella typhimurium (O antigens 4,5,12) or Salmonella sp. serotype dublin (O 9,12) were stimulated with various bacterial cell envelope components, and their [\(^3\)H]thymidine incorporation was measured. It was found that peripheral blood lymphocytes from infected calves incorporated significantly more [\(^3\)H]thymidine than peripheral blood lymphocytes from uninfected controls (\(P\) values ranged from <0.05 to <0.0005). The responder cell type was found in a B-cell-depleted and T-cell-enriched population. The Salmonella infections elicited T-cell responses against at least two cell envelope components: (i) a specific response against the O-antigenic polysaccharide chain of the lipopolysaccharide (This was evident in that a polysaccharide from S. enteritidis [O 9,12] which shares a trisaccharide structure [O antigen 12 determinant] with S. typhimurium stimulated [\(^3\)H]thymidine uptake, which, although lower than in the homologous system, was significantly higher than that seen after incubation with unrelated Salmonella sp serotype thompson polysaccharide.) and (ii) a response against outer membrane proteins (porins), which are present in both S. typhimurium and Salmonella sp. serotype dublin. The experiments with peripheral blood lymphocytes from Salmonella sp. serotype dublin-infected calves gave results in excellent agreement with those obtained in S. typhimurium-infected calves.

Salmonella infections result in stimulation of both humoral and cell-mediated immunity (7). It is nowadays generally accepted that, although humoral immunity contributes to the host defense against Salmonella bacteria, the ability of the components of the cellular immune system to eradicate the invading organism is of overriding importance (14).

Salmonella infections in cattle have often been judged by the presence, or absence, of the bacteria in fecal specimens. Following the infection status with tests detecting cellular immunity has been little used (1, 5). In the accompanying paper (17) we describe the use of a skin test for assessing the delayed type of reactivity which could be detected in calves experimentally infected with Salmonella typhimurium, and the data show a specificity against the O-antigenic polysaccharide (PS) chain of the lipopolysaccharide (LPS).

In this communication we analyze, using in vitro studies, the uptake of [\(^3\)H]thymidine in peripheral blood lymphocytes (PBL) collected from S. typhimurium- and Salmonella sp. serotype dublin-infected calves and uninfected controls when stimulated with various purified structural entities (LPSs, PSs, and porins) of the cell envelopes of S. typhimurium, Salmonella sp. serotype dublin, and Salmonella sp. serotype thompson.

MATERIALS AND METHODS

Bacterial strains. S. typhimurium SH4809 (O antigens 4,5,12), S. enteritidis SH1262 (O 9,12), and Salmonella sp. serotype thompson IS40 (O 6,7) were available from previous investigations (J. Å. Robertsson, S. B. Svensson, L. H. M. Renström, and A. A. Lindberg, Res. Vet. Sci., in press). S. typhimurium SVA44 (O 4,5,12) and Salmonella sp. serotype dublin SVA47 (O 9,12) were from the strain collection of the National Veterinary Institute, Uppsala, Sweden. The SVA44 and SVA47 strains were originally isolated from infected cattle.

Crude extracts, LPSs, O-PSs, and porins from Salmonella spp. Crude extracts of S. typhimurium SVA44 and Salmonella sp. serotype dublin SVA47 were available from previous work (J. Å. Robertsson, S. B. Svensson, and L. H. M. Renström, Res. Vet. Sci., in press). LPSs and O-PSs from S. typhimurium SH4809, S. enteritidis SH1262, and Salmonella sp. serotype thompson IS40 were prepared and characterized as described before (10, 13, 17). Outer membrane pro-
teins (porins) from *S. typhimurium* SL1909 were prepared and characterized as before (16, 19).

**Experimental animals and infection procedures.** Calves (male and female), 5 to 6 weeks old (60 to 70 kg), were orally infected with approximately 10^8 *S. typhimurium* bacteria (strain SVA44) or 10^6 *Salmonella* sp. serotype *dublin* bacteria (strain SVA47). The infection procedure and holding of the calves were as described in the accompanying paper (17). The system for numbering calves is the same in this and the accompanying paper (17); thus, the calves can be identified in both papers.

Transfer of serum from two calves experimentally infected with *S. typhimurium* SVA44 to two control calves was carried out as described in the accompanying paper (17). Two hours after the intravenous injection of serum, the control calves were bled for lymphocyte stimulation tests.

Clinical skin test and bacteriological examinations were done as in the accompanying paper (17).

Humoral antibody titers in infected and uninfected calves were estimated as in the accompanying paper (17).

**Isolation of blood lymphocytes.** Blood was collected in bottles in which heparin (10 IU/ml) had been added, and PBLs were isolated by the Ficoll-Isopaque method of Böyum (3) as applied to bovine blood by Johnson and Morein (11). The viability of the PBLs was determined and was found to be 95% or more by the trypan blue exclusion test (15). The cell concentration was adjusted to 4 × 10^7 viable cells per ml in RPMI 1640 medium supplemented with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (GIBCO Europe, Glasgow, Scotland), 2 mM L-glutamine, 10% fetal calf serum (GIBCO Europe), penicillin (200 IU/ml), and streptomycin (200 μg/ml).

**Detection of immunoglobulin-bearing cells by immunofluorescence technique.** Immunoglobulin-bearing lymphocytes (B-lymphocytes) were detected with fluorescein isothiocyanate-conjugated F(ab')_2 fragments of rabbit anti-bovine immunoglobulin M (IgM) (15). Before being labeled for immunoglobulin, the PBL population was incubated in serum-free medium for at least 2 h at 37°C to remove cytophilic antibodies (20). After being washed, the lymphocytes were incubated with the fluorescein isothiocyanate conjugate for 30 min at 4°C. The cells were washed free from nonbound conjugate, fixed for 5 min, using 1.8% paraformaldehyde, mounted on a glass slide, and examined. The proportion of immunoglobulin-bearing cells was determined by counting at least 200 cells.

**Removal of immunoglobulin-bearing lymphocytes.** B-lymphocytes were removed from the total cell population (PBLs) by affinity fractionation. The method of Wigzell (21) for separation of mouse lymphocytes, modified by Morein et al. (15) for bovine lymphocytes, was used. Briefly, the lymphocyte suspension was passed through a column of glass beads (mean size, 0.2 mm) coated with rabbit immunoglobulin-anti-bovine immunoglobulin complexes as described by Morein et al. (15). Cells with immunoglobulin molecules or Fc receptors (or both) (mostly B-lymphocytes) were retained on the column, and a fraction depleted of these cells (PBL-d) was collected and used in the LS test. The PBL-d populations were examined for B-lymphocytes by the immunofluorescence method described above and were uniformly found to contain <2% B-lymphocytes.

**Lymphocyte stimulation test.** Microplates with well volumes of 0.27 ml and conical bottoms (Sterilin, Ltd., Teddington, England) were used for culturing and for stimulation of the lymphocytes. Approximately 4 × 10^5 cells per well were cultured with 0.1 ml of each antigen preparation diluted in supplemented RPMI medium. The LPS and PS preparations were initially tested in 10-fold dilution steps in concentrations ranging from 0.005 to 500 μg/ml. The concentrations of SVA44 and SVA47 bacteria were used in all reported studies. The crude extract was also used in a concentration of 10 μg/ml. The ability of the lymphocytes to respond to mitogen was tested by incubation with 5 μg of phytohemagglutinin (Pharmacia; Uppsala, Sweden). A stimulation time of 4 days was used in all of the experiments after we compared the uptakes for 4, 7, and 10 days in a pilot study. The cells were pulsed by the addition of 25 μl of supplemented RPMI 1640 medium containing 1 μCi of [3H]thymidine (Kebo-Grave, 185 MBO-3; Spånga, Sweden) to each well. The cells were harvested 24 h later, using a semiautomatic multiple cell culture harvester (Skatron TLX 18625; made for Flow Laboratories, Solna, Sweden), on filter papers which were dried and transferred to vials containing 5 ml of scintillation liquid (Insta-Fluor, 6013027; Packard Instrument Co., Chicago, III.). Each sample was counted in a liquid scintillation counter (Tri Carb, 3255; Packard Instrument Co.). Each antigen was tested in four wells. The highest and lowest values were discarded. Each control consisted of six wells, and again, the highest and lowest values were discarded. The SI was calculated according to the following formula. SI = mean counts per minute of the two wells stimulated with antigen/mean counts per minute of four control wells. SIs were calculated, using 10^log of the individual SI figures (experimental versus control) due to their normal distribution. The [3H]thymidine uptake in PBLs which had not been stimulated with any antigen varied from 314 to 2,505 cpm, with a mean value of 793 cpm (standard deviation, 716 cpm).

**RESULTS**

Male and female calves were the same as those reported in the accompanying communication (17). An abbreviated version of the skin testing results, using various antigenic preparations, in calves infected orally with either 10^4 viable *S. typhimurium* (O 4,5,12) SVA44 or 10^6 viable *Salmonella* sp. serotype *dublin* (O 9,12) SVA47 bacteria is given in Table 1. Calves were infected on day 0 and examined daily for fecal excretion of the infecting organism. Virtually all of the calves excreted the *Salmonella* strain daily during the first 3 weeks after challenge and intermittently thereafter. The skin testing was done on days 31 to 35.

*S. typhimurium*-infected calves. PBLs were collected from six infected and four noninfected
<table>
<thead>
<tr>
<th>Calves (no.) infected with:</th>
<th>Increase in double skin fold thickness (mm) 48 h after intracutaneous injection of different antigen preparations</th>
<th>ELISA titersa x 10⁻³ against:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. typhimurium SVA44 crude extract*</td>
<td>S. typhimurium SL1907 porin*</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>10</td>
<td>5</td>
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<tr>
<td>S. typhimurium</td>
<td>11</td>
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<td>S. typhimurium</td>
<td>15</td>
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<tr>
<td>Mean double skin fold thickness increase (mm)</td>
<td>5.8</td>
<td>1.7</td>
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<tr>
<td>Salmonella sp. serotype dublin</td>
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<td>3</td>
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<tr>
<td>Salmonella sp. serotype dublin</td>
<td>22</td>
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<tr>
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<td>Uninfected calves</td>
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<td>1</td>
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<tr>
<td>Mean double skin fold thickness increase (mm)</td>
<td>1.5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

a The enzyme-linked immunosorbent assay (ELISA) titers are expressed as endpoint titers, i.e., the reciprocal of the serum dilution which gives an absorbance of 0.1 at 405 nm in 100 min.

b The antigen dose injected was 50 μg of protein as determined by Lowry et al. (12).

c The antigen dose injected was 50 μg of hexose as determined by the phenol-sulfuric acid method (8), with D-glucose as the standard.

d ND, Not done.
FIG. 1. \[^{[H]}\]Thymidine uptake in PBLs from *S. typhimurium*-infected and -uninfected calves stimulated with various antigens. Each bar shows the mean of 10\(^{10}\) log values of SIs, with standard deviations indicated. Statistical analyses were made, using Student's *t* test (4). Levels of statistic significances: A-a, 0.05 < *P* < 0.1; A'-a', 0.01 < *P* < 0.025; A''-a'', *P* < 0.0005; B-b, 0.25 < *P*; B'-b', 0.1 < *P* < 0.25; B''-b'', 0.25 < *P*; C-c, 0.25 < *P*; C'-c', 0.0025 < *P* < 0.005; C''-c'', 0.05 < *P* < 0.1; D-d, D'-d', and D''-d'', 0.25 > *P*; E-e, E'-e', and E''-e'', 0.25 < *P*; F-f, 0.1 < *P* < 0.25; F'-f', 0.001 < *P* < 0.0025; F''-f'', 0.05 < *P* < 0.1; G-g, 0.1 < *P* < 0.25; G'-g', 0.05 < *P* < 0.1; G''-g'', 0.25 < *P*; H-h, H'-h', and H''-h'', 0.25 < *P*; I-i, 0.25 < *P*; I'-i', 0.01 < *P* < 0.025; I''-i'', 0.05 < *P* < 0.1.

(control) calves on days 0, 15, and 30, and their uptake of \[^{[H]}\]thymidine was observed after they were stimulated with various different antigen preparations. All PBLs taken on various occasions readily responded to stimulus with phytohemagglutinin, e.g., SI values >5. PBLs collected from *S. typhimurium*-infected calves showed a significantly higher uptake of \[^{[H]}\]thymidine than PBLs from uninfected control calves when incubated with the crude extract from *S. typhimurium* SVA44, which contains LPSs, porins, other outer membrane proteins, and lipoprotein (Fig. 1 and 2). The levels of significance on days 15 and 30 were 0.01 < *P* < 0.025 and *P* < 0.0005, respectively. The level of stimulation caused by the *S. typhimurium* SH4809 (O 4,12) LPS was also significantly higher than that caused by the O-antigenically unrelated *Salmonella* sp. serotype *thompson* IS40 (O 6,7) LPS. Since the LPSs from both of these strains have the same lipid A structure, the results suggested that the stimulation was caused by the PS part of the LPS. The PS preparation from *S. typhimurium* also efficiently stimulated PBLs from the infected as opposed to the noninfected calves, particularly when PBLs were collected 15 days after infection (*P* < 0.005). LPSs and PSs from *S. enteritidis* SH1262 (O 9,12), which shares part of the O-PS repeating units with *S. typhimurium*, e.g., O-antigen 12, were both quite inefficient as stimulators, although slightly more active than the LPSs and PSs from the O-antigenically unrelated *Salmonella* sp. serotype *thompson* IS40 (O 6,7).

Part of the stimulating activity seen with the crude extract could, besides LPS, be ascribed to porins, since the porin fraction caused an increase of \[^{[H]}\]thymidine uptake in infected as compared with control calves (0.01 < *P* < 0.05 and 0.05 < *P* < 0.1, with PBLs collected on days 15 and 30, respectively).

It was evident with all of the *S. typhimurium-
infected calves that the maximum $[^{3}H]$thymidine uptake was seen in PBLs collected on day 15. The mean values for stimulation with LPS, PS, and porins decreased 36, 33, and 24%, respectively, for PBLs sampled on day 30 as compared with those sampled on day 15. The humoral antibody response, measured by enzyme-linked immunosorbent assay, with S. typhimurium SH4809 LPS as antigen and with an alkaline phosphatase-labeled rabbit anti-bovine immunoglobulin conjugate, in infected calves developed gradually, as expected. The titers on days 30 to 35 were 10-fold higher than those on day 15, with mean titers of 50,000 and 3,500, respectively.

**Salmonella sp. serotype dublin-infected calves**. PBLs collected from the three *Salmonella* sp. serotype dublin (O 9,12) SVA47-infected calves were incubated with crude extract, LPS, PS, and porins in the presence of $[^{3}H]$thymidine, as were PBLs from uninfected control calves (Table 2). It could be shown that in *Salmonella* sp. serotype dublin-infected calves, as in *S. typhimurium*-infected calves, the homologous LPS and PS in a highly significant way ($P$ values in the range of $<0.05$ to $<0.005$) stimulated the $[^{3}H]$thymidine uptake in PBLs from infected calves as compared with those from uninfected controls. Also, the porin preparation stimulated uptake, although less efficiently than the LPS and PS preparations (Table 2). The maximum uptake was seen in PBLs collected on day 30; the values for three of the antigens were from 23 to 31% higher than those for PBLs collected on day 15. In one instance, when the porin preparation was used, the SI value on day 30 was 10% lower than that on day 15.
When the SI values for *S. enteritidis* SH1262 LPS and PS were compared with those for the heterologous *Salmonella* sp. serotype thompson IS40 LPS and PS, using PBLs from *Salmonella* sp. serotype dublin SVA47-infected calves, the observed differences were significant, particularly on day 30. When the same comparison was made substituting the partly related LPS and PS preparations from *S. typhimurium* SH4809 for *S. enteritidis* SH1262 LPS and PS preparations, the differences were somewhat smaller. We surmise that this is because the common O antigen 12 antigenic determinant in the PSs from *S. enteritidis* and *S. typhimurium* is a determinant involved in the stimulation.

**Stimulation of PBLs deprived of the immunoglobulin-bearing cells.** The stimulating activity of the various antigens was subsequently tested on PBL populations which had been deprived of their immunoglobulin-bearing B-lymphocytes by passage over a glass bead column coated with rabbit anti-bovine immunoglobulin (PBL-d). The PBLs were collected from *S. typhimurium*-infected calves. After passage, <2% of the lymphocytes were B-cells, as estimated by immunofluorescence. The control population, only two uninfected calves, was so small that in the statistical analyses pooled data (from days 0, 15, and 25) were used.

The PBL-d subpopulations from the infected calves responded with a significantly higher [3H]thymidine uptake than those collected from noninfected control calves (Table 3). LPS and PS preparations from *S. typhimurium* were efficient antigens, as was the porin preparation (Fig. 2). The maximal stimulation seen after 15 days was the same as that for the experiments with *S. typhimurium* reported above (Fig. 1 and 2). The SI values for the PBL population when tested before removal of the B-cells did not differ significantly from those seen with the PBL-d population (0.25 < *P*) (data not shown).

**Calves passively transferred with anti-*S. typhimurium* serum.** Cell-free sera were collected on day 37 from two *S. typhimurium*-infected calves (no. 17 and 18) and injected intravenously into uninfected control calves (no. 39 and 40). No stimulation could be seen in PBLs collected from calves 39 and 40 2 h after transfusion; [3H]thymidine uptake after stimulation with crude extract and LPS, PS, and porin preparations were ≤0.18. The antibody titers against the *S. typhimurium* LPS antigen were also estimated in sera collected 2 h after transfusion. Relative titers were 15,000 and 10,000, respectively, as compared with <500 in both calves before transfusion.

**DISCUSSION**

The in vivo immune reactivity, seen as specific delayed skin swellings in *S. typhimurium-*
infected calves intradermally inoculated with LPS and a porin preparation from *S. typhimurium* (17), could also be observed in in vitro assays, using [3H]-thymidine uptake in stimulated PBLs. When the PBLs from infected calves were incubated with a crude extract (containing LPS, porins, and other outer membrane components) or purified components such as LPS, O-antigenic PS, or porins from *S. typhimurium*, the incorporation of [3H]-thymidine was significantly higher than in PBLs from uninfected control calves (from $P < 0.05$ to $P < 0.005$), using PBLs collected 15 days after infection (Fig. 1). It is evident that a significant portion of the activity seen with the crude extract could be ascribed to the O-PS chain of the LPS (Fig. 1). The O-PS chain also contributed to the specificity of the reaction, whereas the porin was as reactive with PBLs from *Salmonella* sp. serotype *dublin* (O 9,12)-infected calves as with PBLs from *S. typhimurium*-infected calves (Fig. 1, Table 2).

PBLs which had been deprived of immunoglobulin-carrying lymphocytes, i.e., B-cells, were highly stimulated by both the PS and the porin preparations (Table 3). This further suggests a T-cell-mediated immune reactivity in *Salmonella*-infected cattle.

The specificity of the O-antigenic PS from *S. typhimurium* SH4809 used as antigen was evident when its stimulating activity was compared with that of the totally unrelated PS from *Salmonella* sp. serotype *thompson* ($P < 0.005$ on day 15). The fact that the difference between the *S. typhimurium* and *S. enteritidis* PS preparations was smaller when the uptakes were compared ($0.05 < P < 0.1$) is not surprising. The PSs share the → 2-D-Man 1→ 4-L-Rha 1→ 3-D-Gal 1→ trisaccharide structure in the tetrasaccharide repeating unit of the O-PS chain, and this structure is responsible for the O antigen 12 antigenic determinant(s) common to these PSs. The same tendency was evident when the two PS antigens were used with PBLs from *Salmonella* sp. serotype *dublin* (O 9,12)-infected calves (Table 2).

Porins are hydrophobic outer membrane proteins and are difficult to obtain free from LPS. It could therefore be argued that the increased SI values seen with porin as antigen and when incubated with PBLs from *S. typhimurium*-infected calves was a consequence of the amount of contaminating LPS (<10% of the material) in the porin preparation. Two facts make such an interpretation highly unlikely. First, the porin was extracted from a rough mutant of *S. typhimurium* which lacks the O-PS chain, and second, the porin preparation stimulated lymphocytes from *Salmonella* sp. serotype *dublin*-infected calves as efficiently as those from *S. typhimurium*-infected calves (Fig. 1, Table 2).

The slightly higher SI values seen when lym-
phocytes from uninfected calves were stimulated with crude extract and LPS as compared with PS (Fig. 1) can be ascribed to the polyclonal activation of B-lymphocytes brought about by the lipid A moiety of the LPS (2).

The results of the in vivo and in vitro experiments in the accompanying paper (17) and in this communication are in accordance. However, the PS preparation failed to elicit a delayed skin reactivity in S. typhimurium-infected calves, but readily stimulated [3H]-thymidine uptake in lymphocytes collected from these calves. The reason for this discrepancy is not clear, but we surmise that development of the delayed skin reaction is more complex than the uptake of thymidine measured in vitro (see accompanying paper for a more detailed discussion). It is also likely that the high solubility of the O-PS allows it to rapidly diffuse away from the inoculation site, thereby not maintaining a local O-antigen epitope density high enough to sustain a delayed skin reaction (18). It is noteworthy that such diffusion does not occur in the in vitro proliferation assays.

The possibility that contaminating trace amounts of protein(s) in the LPS and saccharide preparations were responsible for the stimulation of the thymidine uptake instead of the PS is considered unlikely based on the following facts: (i) The O-PS from S. typhimurium SH4809 contained ≤1% protein as estimated by the method of Lowry et al. (12) and had, during its preparation, been boiled for hours in 0.15 M sodium hydroxide-1% acetic acid (17). (ii) The octasaccharide was obtained by enzymatic hydrolysis followed by dialysis through a membrane with an exclusion limit of molecular weight 8,000 for globular proteins, and it was subsequently purified by gel chromatography and by high-powered liquid chromatography and found to have a molecular weight of 1,250 (16). In these assays and by 1H- and 13C-nuclear magnetic resonance spectrometry, no evidence whatsoever was found for contaminating proteins. (iii) The specificity found in the [3H]-thymidine uptake assays could not be a consequence of proteins shown to be the same for Salmonella sp. (9), but must rest with the structure of the O-PS. (iv) In a titration of the amount of porins required to give a significant uptake of [3H]-thymidine in PBLs from S. typhimurium-infected calves, the 1.0-µg dose gave the following SI values in mean and (standard deviation): day 0, -0.02 (0.09); day 15, 0.01 (0.07); and day 30, 0.07 (0.08). These values are considerably lower than those seen for the PS preparations (Fig. 1 and 2, Table 3). Since the concentration of PS used in the stimulation experiments was 10.0 µg/ml, a protein contamination exceeding 10% would be required to cause the significant uptake seen. The analytical data exclude such an interpretation.

It would be desirable to be able to show that the immunity could be passively transferred with sensitized T-cells. Practical complications working with calves have so far made such experiments nonfeasible.

We feel confident in stating that the present results show that bovine PBLs from infected calves can respond to purified PS chains or porins from relevant Salmonella strains by thymidine incorporation. To our knowledge this has not been reported previously. The responder cell type is found in a B-cell-depleted and a T-cell-enriched population as demonstrated by the enhanced ability of PBLs passed through an immunoglobulin-anti-immunoglobulin column to respond. That the PS molecules indeed can induce such a T-cell response is also strongly suggested, first, by the fact that the PS preparations are extremely pure (17), and second, by the fact that the cross-reactions observed when LPS and PS preparations from various Salmonella strains were used as antigens correlated well with known molecular features of the PSs e.g., shared structural domains. It should be realized, however, that the present tests do not allow the conclusion to be drawn that the PSs directly can trigger T-cells, as this normally occurs via interactions with cell membranes of antigen-presenting cells (6). We surmise that the cell-mediated immune response is elicited as a consequence of the ability of the lipid portion of the LPS to adhere and insert into mammalian cell membranes (17). In that position it is likely that LPSs are recognized as foreign in the way virus-encoded glycoproteins are recognized as foreign in virus-infected mammalian cells.

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LITERATURE CITED


