

## Phase 1 and Phase 2 Studies of *Salmonella enterica* Serovar Paratyphi A O-Specific Polysaccharide-Tetanus Toxoid Conjugates in Adults, Teenagers, and 2- to 4-Year-Old Children in Vietnam

EDWARD Y. KONADU,<sup>1</sup> FENG-YING C. LIN,<sup>1</sup> VÔ ANH HÓ,<sup>2</sup> NGUYEN THI THANH THUY,<sup>3</sup>  
PHAN VAN BAY,<sup>2</sup> TRAN CONG THANH,<sup>3</sup> HA BA KHIEM,<sup>3</sup> DANG DUC TRACH,<sup>4</sup>  
ARTHUR B. KARPAS,<sup>1</sup> JIANPING LI,<sup>1</sup> DOLORES A. BRYLA,<sup>1</sup>  
JOHN B. ROBBINS,<sup>1</sup> AND SHOUSUN C. SZU<sup>1\*</sup>

National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-2720,<sup>1</sup>  
and Huu Nghi Hospital, Cao Lãnh District, Dong Thap Province,<sup>2</sup> Pasteur Institut, Ho Chi Minh City,<sup>3</sup>  
and National Institute of Hygiene and Epidemiology, Hanoi,<sup>4</sup> Vietnam

Received 23 July 1999/Returned for modification 27 October 1999/Accepted 26 November 1999

*Salmonella enterica* serovar Paratyphi A O-specific polysaccharide (O-SP) was activated with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) and bound to tetanus toxoid (TT) with adipic acid dihydrazide as a linker (SPA-TT<sub>1</sub>) or directly (SPA-TT<sub>2</sub>). In mice, these two conjugates elicited high levels of immunoglobulin G (IgG) anti-lipopolysaccharide (LPS) in serum with bactericidal activity (E. Konadu, J. Shiloach, D. A. Bryla, J. B. Robbins, and S. C. Szu, *Infect. Immun.* 64:2709–2715, 1996). The safety and immunogenicity of the two conjugates were then evaluated sequentially in Vietnamese adults, teenagers, and 2- to 4-year-old children. None of the vaccinees experienced significant side effects, and all had preexisting LPS antibodies. At 4 weeks after injection, there were significant increases of the geometric mean IgG and IgM anti-LPS levels in the adults and teenagers: both conjugates elicited a greater than fourfold rise in the IgG anti-LPS level in serum in ≥80% of the volunteers. SPA-TT<sub>2</sub> elicited slightly higher, though not statistically significantly, levels of IgG anti-LPS than did SPA-TT<sub>1</sub> in these age groups. Accordingly, only SPA-TT<sub>2</sub> was evaluated in the 2- to 4-year-old children. On a random basis, one or two injections were administered 6 weeks apart to the children. No significant side effects were observed, and the levels of preexisting anti-LPS in serum were similar in children of all ages. A significant rise in the IgG anti-LPS titer was elicited by the first injection ( $P = 0.0001$ ); a second injection did not elicit a booster response. Representative sera from all groups had bactericidal activity that could be adsorbed by *S. enterica* serovar Paratyphi A LPS.

Enteric fever, with its septicemia and complications, is caused by *Salmonella* serogroups A, B, C, and D. Although reported throughout the world several decades ago (2, 13), *Salmonella enterica* serovar Paratyphi A seems to be confined to Southeast Asia, where it is the second most common cause of enteric fever, accounting for about 10% of cases (1, 3, 19, 22, 25, 28, 29, 31, 36, 37, 39–43, 46–48, 55, 57, 62, 63, 66). Despite the frequency and severity of enteric fevers and efforts to control the diseases, there is no licensed vaccine for nontyphoidal salmonellae. TAB vaccine, composed of inactivated *Salmonella*, was removed as a licensed product because efficacy could not be verified for groups A and B (17, 20, 26, 59).

Both *S. enterica* serovars Typhi and Paratyphi are inhabitants and pathogens of humans only, and they can be considered clones (11, 21, 24, 32, 45, 49, 53, 58). The lipopolysaccharide (LPS) of nontyphoidal *Salmonella* is both an essential virulence factor and a protective antigen (50). Animal experiments on the structurally related serogroup B and clinical results with Vi capsular polysaccharide of *S. enterica* serovar Typhi were the basis for our prediction that a critical level of serum immunoglobulin G (IgG) to the O-specific polysaccharide (O-SP) will confer protection against *S. enterica* serovar

Paratyphi A (10, 15, 16, 50, 58, 64). Accordingly, we developed O-SP conjugates of *S. enterica* serovar Paratyphi A designed to elicit IgG LPS antibodies in serum (35).

The O-SP of *S. enterica* serovar Paratyphi A is composed of a trisaccharide backbone with a branch of D-paratose from the C-3 of  $\alpha$ -D-mannose; C-3 of the adjacent  $\alpha$ -L-rhamnose is partially O acetylated (8, 9, 23, 27) (Fig. 1).

*S. enterica* serovar Paratyphi A O-SP required O-acetyl groups to elicit serum LPS antibody with bactericidal activity in mice (33). O-SP was activated with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) at neutral pH and bound either directly or through an adipic acid hydrazide linker to the protein (33, 35, 38). In mice, *S. enterica* serovar paratyphi A conjugates induced LPS antibody with group-specific bactericidal activity against serogroup A.

We describe a clinical evaluation of *S. paratyphi* A O-SP, bound to tetanus toxoid (TT) directly or through an adipic acid hydrazide spacer, in adults, teenagers, and 2- to 4-year-old children.

### MATERIALS AND METHODS

**Investigational vaccines.** The O-SP was prepared as described previously and had a molar ratio of 0.71 O-acetyl groups per repeating unit of polysaccharide (33). Pasteur-Mérieux Serum et Vaccins, Lyon, France, provided TT (lot GYA). Conjugates of the O-SP of *S. enterica* serovar Paratyphi A bound to TT through a linker, adipic acid dihydrazide (SPA-TT<sub>1</sub>; lot 64811), or directly (SPA-TT<sub>2</sub>, lot 64812) have been described previously (13, 35). SPA-TT<sub>1</sub> contained 30.2  $\mu$ g of protein per ml and 48.6  $\mu$ g of polysaccharide per ml, and SPA-TT<sub>2</sub> contained 40.7  $\mu$ g of protein per ml and 51.3  $\mu$ g of polysaccharide per ml; the dose for both

\* Corresponding author. Mailing address: National Institute of Child Health and Human Development, National Institutes of Health, Bldg. 6, Room 424, Bethesda, MD 20892. Phone: (301) 496-4524. Fax: (301) 402-9108. E-mail: scszu@helix.nih.gov.

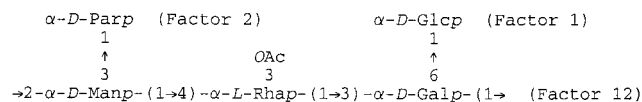


FIG. 1. Structure of *S. enterica* serovar Paratyphi O-SP.

conjugates was 0.5 ml. Both lots passed the Food and Drug Administration requirements for sterility, pyrogenicity, and general safety.

**Study protocol.** The investigation was approved by the Ministry of Health of Vietnam; the Institutional Review Board of the National Institute of Child Health and Human Development, National Institutes of Health (protocol OH96-CH-N023); the Office of Protection from Research Risks; and the Food and Drug Administration (BB IND 6530). Informed consent was obtained from adult volunteers and from parents or guardians of vaccinees younger than 18 years.

The site was Cao Lãnh District, Dong Thap Province, of the Mekong Delta region of southern Vietnam. Twenty healthy adults were randomly assigned to receive one 0.5-ml injection of either SPA-TT<sub>1</sub> or SPA-TT<sub>2</sub> in the deltoid muscle. The vaccinees were examined at 30 min and at 6, 24, and 48 h following immunization. Reactions at the immunization sites were inspected, and the temperatures of the volunteers were measured; these findings were recorded. Side effects were defined as a fever of >38.5°C, erythema of >2.5 cm, or induration of >2 cm within 48 h of the injection. Sera were obtained before and at 6 and 26 weeks after vaccination and stored at -70°C. When it was confirmed that adults did not experience significant side effects, 108 teenagers between 13 and 17 years old were injected with one of these two conjugates according to the same protocol. The IgG LPS antibody titers in serum in the two groups were assayed and SPA-TT<sub>2</sub>, which elicited higher geometric mean (GM) IgG LPS antibody levels in serum, was chosen for evaluation in the 2- to 4-year-old children. A total of 110 children between 2 and 4 years old were injected with SPA-TT<sub>2</sub>. Half of the children were randomly assigned to receive a second dose 6 weeks later. Vaccinees were observed for adverse reactions at 30 min and 6, 24, and 48 h after each injection. Sera were collected before each injection and at 6, 10, and 26 weeks after the first injection.

**LPS antibodies in serum.** Sera were analyzed for IgG and IgM LPS antibodies by enzyme-linked immunosorbent assay (ELISA) (35). Goat anti-human IgG or IgM conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was the secondary antibody. The results are expressed in ELISA units (EU) as a percentage of a high-titer serum from an adult vaccinee. A response was defined as at least a fourfold rise in titer compared to the level at time zero. Bactericidal activity was assayed as described previously (34). Antibodies to TT were measured by ELISA with a human tetanus immune globulin, titrated for its neutralizing activity, as a reference (14). Results for TT antibodies are expressed in antitoxin units.

**Statistical analyses.** All antibody levels are expressed as GM. Levels below the sensitivity of the ELISA (0.1 EU) were assigned the value of one-half of that level (0.05 EU). Comparison of GMs was performed by either a paired or unpaired *t* test or the Wilcoxon test when appropriate.

**RESULTS**

**Clinical observations.** No significant side effects were reported for any of the three age groups.

**LPS antibody levels in serum. (i) Prevacination.** All vaccinees had preexisting IgG and IgM antibodies in serum. The

adults and 13- to 17-year-old teenagers had similar levels that were significantly higher than those in the 2- to 4-year-old children (1.46, 1.70, and 0.85 EU, respectively, for IgG [*P* < 0.05]; 17.5, 22.6, and 6.80, EU, respectively, for IgM [*P* = 0.0001]).

**(ii) Adults.** At 6 weeks after injection, 75% of the adults responded with at least a fourfold rise in the IgG anti-LPS titer (Table 1). Recipients of SPA-TT<sub>2</sub> had higher IgG anti-LPS levels than did recipients of SPA-TT<sub>1</sub> (27.3 and 9.82 EU, respectively [not significant]) at 42 days following injection. These levels fell about threefold at 180 days but were still higher than the prevaccination levels (8.28 versus 1.69, and 3.73 versus 1.26 EU [*P* < 0.005]). Recipients of SPA-TT<sub>2</sub> had higher levels of both IgG and IgM than did recipients of SPA-TT<sub>1</sub> (8.28 and 3.73 EU, respectively [*P* = 0.03]).

Although significant, rises in the IgM anti-LPS levels induced by SPA-TT<sub>1</sub> (35.2 versus 17.3 EU [*P* = 0.0004]) and by SPA-TT<sub>2</sub> (42.3 versus 17.7 EU [*P* = 0.004]) 6 weeks after injection were lower than that in the IgG level. IgM anti-LPS antibody levels elicited by SPA-TT<sub>2</sub> were only slightly higher than those elicited by SPA-TT<sub>1</sub> (42.3 and 35.2 EU, respectively [*P* > 0.05]). IgM anti-LPS levels at the 180-day interval declined to levels similar to those prior to the vaccination.

**(iii) Teenagers.** Conjugate-induced IgG anti-LPS levels in the 13- to 17-year-old volunteers were similar to but slightly lower than those in the adults (Table 1). At the 6-week interval, 85% responded with at least a fourfold rise in the IgG anti-LPS titer (*P* = 0.0001). Recipients of SPA-TT<sub>1</sub> had a 7.6-fold rise to 12.8 EU, and recipients of SPA-TT<sub>2</sub> elicited a 10.2-fold rise to 17.4 EU (*P* > 0.01). At 180 days after vaccination, the IgG anti-LPS titers had declined about 50% compared to those at 42 days: the level elicited by SPA-TT<sub>2</sub> was only slightly higher than that elicited by the SPA-TT<sub>1</sub> (7.37 and 6.72 EU, respectively, *P* > 0.05). These levels were higher than the preimmune levels (6.72 versus 1.69, and 7.37 versus 1.70 EU [*P* = 0.0001]).

As with IgG anti-LPS, the IgM responses in serum elicited by the two conjugates were similar to those observed in the adults. At the 42-day interval, the IgM anti-LPS titer had risen 1.4-fold (*P* = 0.0001) in the SPA-TT<sub>1</sub> group and 1.5-fold (*P* = 0.0001) in the SPA-TT<sub>2</sub> group. At 180 days, the IgM anti-LPS levels in the teenagers and adults were similar to each other and to the prevaccination levels.

Based on these results, only SPA-TT<sub>2</sub> was evaluated in 2- to 4-year-old children.

**(iv) Children.** About 90% of the children had at least a fourfold rise in their IgG anti-LPS titer at the 42-day interval

TABLE 1. LPS antibodies elicited by SPA-TT conjugates in sera of adults and teenagers

| Ig  | Conjugate           | Age (yr) | No. | GM LPS antibody titer <sup>a</sup> (25th to 75th percentiles) (EU) on days after vaccination: |                  |                  |
|-----|---------------------|----------|-----|---|------------------|------------------|
|     |                     |          |     | 0   | 42               | 180              |
| IgG | SPA-TT <sub>1</sub> | 18-44    | 10  | 1.26 (0.6-2.6)  | 9.82 (4.7-15.6)  | 3.73 (2.67-7.10) |
|     | SPA-TT <sub>2</sub> | 18-44    | 10  | 1.69 (1.0-2.9)  | 27.3 (8.65-54.1) | 8.28 (9.86-10.1) |
|     | SPA-TT <sub>1</sub> | 13-17    | 56  | 1.69 (0.98-2.69)  | 12.8 (6.75-22.9) | 6.72 (4.25-11.6) |
|     | SPA-TT <sub>2</sub> | 13-17    | 52  | 1.70 (1.13-2.34)  | 17.4 (10.3-22.6) | 7.37 (4.80-12.0) |
| IgM | SPA-TT <sub>1</sub> | 18-44    | 10  | 17.3 (8.32-31.5)  | 35.2 (23.7-48.9) | 18.7 (11.9-39.5) |
|     | SPA-TT <sub>2</sub> | 18-44    | 10  | 17.7 (11.3-26.4)  | 42.3 (25.4-74.9) | 24.1 (16.4-30.6) |
|     | SPA-TT <sub>1</sub> | 13-17    | 56  | 20.7 (14.3-28.0)  | 29.6 (20.6-37.9) | 21.9 (17.2-25.4) |
|     | SPA-TT <sub>2</sub> | 13-17    | 52  | 24.9 (17.8-32.5)  | 37.3 (27.1-48.6) | 23.9 (21.4-28.9) |

<sup>a</sup> Serum standard is from a high-responder vaccinee and is assigned a value of 100 EU.

TABLE 2. LPS antibodies in serum of children receiving one or two injections of SPA-TT<sub>2</sub>

| Ig  | No. of injections | No. of children | GM LPS antibody titer <sup>a</sup> (25th to 75th percentiles) (EU) on days after vaccination: |                  |                  |                  |
|-----|-------------------|-----------------|---|------------------|------------------|------------------|
|     |                   |                 | 0   | 42 <sup>b</sup>  | 70               | 180              |
| IgG | 1                 | 63              | 0.91 (0.49–1.56)  | 19.3 (8.40–38.1) | 11.7 (6.13–21.4) | 3.47 (2.34–4.79) |
|     | 2                 | 47              | 0.77 (0.47–1.17)  | 16.7 (9.41–32.3) | 11.9 (6.57–19.8) | 4.08 (2.59–6.00) |
| IgM | 1                 | 63              | 6.65 (3.52–11.4)  | 28.8 (16.3–50.4) | 22.0 (13.6–41.8) | 15.9 (10.1–21.3) |
|     | 2                 | 47              | 7.00 (4.67–10.3)  | 32.3 (16.3–48.3) | 24.7 (13.6–44.9) | 17.6 (10.3–26.2) |

<sup>a</sup> A high-responder serum was used as the standard and assigned a value of 100 EU.

<sup>b</sup> Group 2 reinjected after bleeding.

(19.3 versus 0.91, and 16.7 versus 0.77 EU [ $P = 0.0001$ ]) (Table 2). Reinjection at 42 days did not elicit a booster response: the levels 2 weeks after the second injection (11.7 versus 11.9 EU [not significant]) and 180 days after the first interval (4.08 versus 3.47 EU [not significant]) were similar in both groups. The IgG anti-LPS levels at 180 days were higher than at prevaccination (3.47 versus 0.91, and 4.08 versus 0.77 EU [ $P = 0.0001$ ]).

IgM anti-LPS responses were slightly different from those of the teenagers and adults. The rises in IgM anti-LPS titers were slightly higher (ca. fourfold), although the levels at 42 days were similar to those in the teenagers and adults. As with IgG, there was no booster response to a second injection. At 180 days, IgM anti-LPS levels were higher in the children than in the teenagers and adults and were about twofold higher than the prevaccination levels (15.9 versus 6.65, and 17.6 versus 7.00 EU [ $P = 0.0001$ ]).

(v) **Summary.** GM IgG anti-LPS levels in serum in vaccinees of the three age groups are summarized in Table 3. The levels of anti-LPS in the preimmune sera were similar for the adults and teenagers; both of these groups had higher levels than did the children. Both the levels and fold rises elicited by SPA-TT<sub>2</sub> were slightly but not significantly higher than those elicited by SPA-TT<sub>1</sub>. With the exception of SPA-TT<sub>1</sub> in the adults, there was a gradual decline in the IgG anti-LPS levels at 180 days that was age related.

**Serum bactericidal levels.** Representative sera (10) from the three age groups were assayed for their bactericidal titer (Table 4). Two pairs of sera from the children, prevaccination and after the first vaccination, were assayed, and their bactericidal titers were compared to the IgG and IgM anti-LPS titers by ELISA. All these sera had a bactericidal activity that required complement. The bactericidal activity was roughly related to the IgG anti-LPS titer. Adsorption of these sera with the LPS

of *S. enterica* serovar Paratyphi A removed most of the bactericidal activity (data not shown).

**Anti-TT levels in serum.** All volunteers had preexisting IgG anti-TT: the children had the highest levels among the three age groups, probably due to their comparatively recent immunization with DTP. The levels at 180 days following injection were similar to those at 42 days in the adults and teenagers. In the children, the anti-TT levels at 180 days declined to about one-third the levels at 42 days but were higher than those in the adults and teenagers (3.62 and 4.10 versus 0.33 and 0.24 for teenagers and 0.48 and 0.56 for adults [ $P = 0.0001$ ]). As observed for LPS antibodies, SPA-TT<sub>2</sub> elicited slightly higher levels and fold rises of TT antibody for all three age groups than SPA-TT<sub>1</sub> did. At 180 days, the children receiving two injections had slightly higher levels of TT antibody than did those receiving only one injection (9.8 and 7.7, respectively [ $P > 0.05$ ]).

DISCUSSION

Enteric fever is a systemic infection caused by *S. enterica* serovar Typhi and salmonellae of groups A to D (21, 32, 53). We proposed that a critical level of IgG LPS antibody in serum could initiate complement-mediated lysis of these pathogens as they enter the intestine (50, 52, 61). Vaccination with the surface polysaccharide of *S. enterica* serovar Typhi (Vi) prevents typhoid fever (65). The immune moiety elicited by Vi is mainly serum IgG antibody (51). The O-SP of *S. enterica* serovar Paratyphi A alone is not immunogenic (hapten) due to its comparatively low molecular weight (30, 35, 60). The failure of whole-cell bacterial vaccines, such as TAB, to confer protection against group A and B salmonellae may be likened to the failure of parenterally injected inactivated gram-negative bacteria to induce high levels of IgG LPS antibodies (6, 18; T. A. Schwartz, D. V. Alcidi, V. Numsuwan, and D. J. Gocke,

TABLE 3. Age-related IgG levels of LPS antibody in serum before and 180 days after vaccination with SPA-TT conjugates

| Age (yr) | Conjugate           | No. of injections | GM LPS antibody titer (EU) on days after vaccination: |      |                 |      | Fold rise at 180 days |
|----------|---------------------|-------------------|---|------|-----------------|------|-----------------------|
|          |                     |                   |   |      |                 |      |                       |
|          |                     |                   | 0   | 42   | 68              | 180  |                       |
| 18–44    | SPA-TT <sub>1</sub> | 1                 | 1.26  | 9.82 | NA <sup>a</sup> | 3.73 | 3.0                   |
|          | SPA-TT <sub>2</sub> | 1                 | 1.69  | 27.3 | NA              | 8.28 | 4.9                   |
| 13–17    | SPA-TT <sub>1</sub> | 1                 | 1.69  | 12.8 | NA              | 6.72 | 4.0                   |
|          | SPA-TT <sub>2</sub> | 1                 | 1.70  | 17.4 | NA              | 7.37 | 4.3                   |
| 2–4      | SPA-TT <sub>2</sub> | 1                 | 0.91  | 19.3 | 11.7            | 3.47 | 3.8                   |
|          | SPA-TT <sub>2</sub> | 2                 | 0.77  | 16.7 | 11.9            | 4.08 | 5.3                   |

<sup>a</sup> NA, not applicable.

TABLE 4. Bactericidal levels elicited by SPA-TT conjugates in sera of vaccinees

| Subject            | Group    | IgG antibody titer (EU) |      | Bactericidal titer |
|--------------------|----------|-------------------------|------|--------------------|
|                    |          | IgG                     | IgM  |                    |
| P1004              | Adult    | 73.6                    | 17.0 | 1:8,000            |
| P1012              | Adult    | 15.5                    | 31.5 | 1:2,000            |
| P2033              | Teenager | 10.1                    | 36.8 | 1:2,000            |
| P2061              | Teenager | 133                     | 73.6 | 1:8,000            |
| P3002              | Child    | 78.8                    | 32.4 | 1:1,600            |
| P3018 <sup>a</sup> | Child    | 1.76                    | 11.4 | 1:500              |
| p3018              | Child    | 118                     | 46.4 | 1:4,000            |
| p3094 <sup>a</sup> | Child    | 0.73                    | 2.73 | 1:500              |
| p3094              | Child    | 58.5                    | 10.8 | 1:8,000            |

<sup>a</sup> Prevaccination sample.

TABLE 5. IgG levels of tetanus antitoxin elicited by SPA-TT conjugates in sera of volunteers

| Age (yr) | Conjugate                        | GM antitoxin level (25th to 75th percentiles) on days after vaccination: |                  |                  |                  | Fold rise at 180 days |
|----------|----------------------------------|--|------------------|------------------|------------------|-----------------------|
|          |                                  | 0  | 42               | 70               | 180              |                       |
| 18-44    | SPA-TT <sub>1</sub>              | 0.29 (0.12-0.59)   | 0.57 (0.23-1.38) | NA <sup>a</sup>  | 0.56 (0.25-1.01) | 1.9                   |
|          | SPA-TT <sub>2</sub>              | 0.15 (0.09-0.16)   | 0.33 (0.11-0.66) | NA               | 0.48 (0.24-0.52) | 3.2                   |
| 13-17    | SPA-TT <sub>1</sub>              | 0.11 (0.07-0.15)   | 0.24 (0.09-0.47) | NA               | 0.24 (0.14-0.36) | 2.2                   |
|          | SPA-TT <sub>2</sub>              | 0.11 (0.07-0.15)   | 0.27 (0.15-0.31) | NA               | 0.33 (0.20-0.50) | 3.0                   |
| 2-4      | SPA-TT <sub>2</sub>              | 0.47 (0.29-0.81)   | 11.5 (8.31-20.8) | 6.73 (4.66-12.7) | 3.62 (2.54-5.19) | 7.7                   |
|          | SPA-TT <sub>2</sub> <sup>b</sup> | 0.42 (0.25-0.73)   | 8.76 (4.89-20.3) | 6.74 (3.61-17.2) | 4.10 (1.71-10.7) | 9.8                   |

<sup>a</sup> NA, not applicable.

<sup>b</sup> Received a second injection on day 42.

Letter, J. Infect. Dis. **158**:1135-1136, 1988). Another explanation might have been gradual hydrolysis of *O*-acetyl groups of the LPSs in TAB vaccine that are essential for eliciting LPS antibody with bactericidal activity to *S. enterica* serovar Paratyphi A (35).

*S. enterica* serovar Paratyphi A is a pathogen of humans only, and we propose that conjugate-induced bactericidal antibodies will provide a correlate for protection (35, 50-52, 61). Subcutaneous injection of mice with *S. enterica* serovar Paratyphi A conjugates using only 1/10 of a human dose in saline elicited bactericidal antibodies specific for group A but not group B *Salmonella* (35). This is consistent with the immunodominant region of the group-specific antigen (factor 2), conferred by paratose, as predicted from the proposed structures of group A, B, and D O-SPs of salmonellae (8-10, 17).

Earlier, we found that *O*-acetyl groups on the O-specific polysaccharide of *S. enterica* serovar Paratyphi A are essential for its immunogenicity and that conjugation with CDAP is useful for synthesis of *S. enterica* serovar Paratyphi A conjugates to preserve *O*-acetyl epitopes (35, 38). Activation by CDAP occurs at neutral pH and does not reduce *O*-acetyls on the O-SP. CDAP-activated O-SP, bound directly to TT without a spacer (SPA-TT<sub>2</sub>), elicited the highest level of TT antibodies ( $P < 0.005$ ) as well as slightly higher level of anti-LPS.

SPA-TT<sub>2</sub> did not elicit a booster response in the 2- to 4-year-old group. This is in contrast to the booster response elicited in this age group by our pneumococcus type 6B-TT, Vi-rEPA, and *Shigella flexneri* 2a-rEPA conjugates (4, 54). There is no explanation for these differing properties of polysaccharide-protein conjugates. We plan to evaluate the *S. enterica* serovar Paratyphi A O-SP conjugate in infants, an age group that shows booster responses to conjugate vaccines (51).

The C-3 of rhamnose of the O-SP is partially *O* acetylated (27). We found a molar ratio of 0.71 *O*-acetyl per repeat unit (35). However, we detected two acetamido signals in the <sup>13</sup>C nuclear magnetic resonance spectrum. The O-SP of *S. typhimurium* (group B) is 2-O acetylated at abeucose, conferring the specificity defined as factor 5 (8, 9). Carlin et al. reported that a murine monoclonal IgA antibody to factor 5 had low protective activity against *S. enterica* serovar Typhimurium (10). It cannot be concluded that the *O*-acetyl does not have important biological activity for group B *Salmonella* because IgA may not protect against blood-borne infection. The failure of de-*O*-acetylated O-SP of *S. enterica* serovar Paratyphi A to induce bactericidal antibodies in mice might be due to the fact that paratose (factor 2 or group-specific antigen) is partially *O* acetylated. Since antibody-mediated immunity to nontyphoidal salmonellae is largely group specific and since the dideoxy sugars are essential for this specificity (8, 9, 17), the location(s)

and immunologic roles of *O*-acetyls in O-SP of groups A, B, and D should be investigated further. *O*-acetyls are essential for expression of the protective action of other polysaccharides (5).

In summary, *S. enterica* serovar Paratyphi A O-specific polysaccharide-TT conjugates were safe and elicited IgG antibodies with bactericidal activity in the serum of adults, teenagers, and 2- to 4-year-old children. There is sufficient information about Vi (65), with *S. enterica* serovar Typhimurium O-SP conjugates in mice (10, 16, 64) and clinical studies with *Shigella* O-SP conjugates (15), to evaluate our *S. enterica* serovar Paratyphi A conjugates for efficacy. If effective, O-SP conjugates against group B, C, and D *Salmonella* should also protect against nontyphoidal enteric fever.

#### ACKNOWLEDGMENTS

We are grateful to Vee Gill and the staff of the Microbiology Branch, Clinical Center, NIH for their assistance. James C. Mond, Uniformed Services University of the Health Sciences, Bethesda, Md., and Andrew Lees, Virion Systems Inc., Rockville, Md., provided helpful advice about CDAP.

#### REFERENCES

- Acharya, G., T. Butler, M. Ho, P. R. Sharma, M. Tawari, R. K. Adhikari, J. B. Khagda, B. Pokhrel, and U. N. Pathak. 1995. Treatment of typhoid fever: randomized trial of a three-day course of ceftriaxone versus a fourteen-day course of chloramphenicol. *Am. J. Trop. Med. Hyg.* **52**:162-165.
- Adeyokunnu, A., and R. G. Hendrickse. 1980. *Salmonella* osteomyelitis in childhood. *Arch. Dis. Child.* **55**:175-184.
- Anand, A. C., V. K. Kataria, W. Singh, and S. K. Chatterjee. 1990. Epidemic multidrug resistant enteric fever in eastern India. *Lancet* **335**:352.
- Ashkenazi, S., J. H. Passwell, E. Harlev, D. Miron, R. Dagan, N. Farzan, R. Ramon, F. Majadly, D. A. Bryla, A. B. Karpas, J. B. Robbins, R. Schneerson, and the Israel Pediatric *Shigella* Study Group. 1999. Safety and immunogenicity of *Shigella sonnei* and *Shigella flexneri* 2a O-specific polysaccharide conjugates in adults. *J. Infect. Dis.* **179**:1565-1568.
- Avery, O. T., and W. F. Goebel. 1933. Chemoimmunological studies on the soluble specific substance of pneumococcus. I. The isolation and properties of the acetyl polysaccharide of pneumococcus type 1. *J. Exp. Med.* **58**:731-755.
- Baumgartner, J. D., D. Heumann, T. Calandra, and M. P. Glauser. 1991. Antibodies to lipopolysaccharide after immunization of humans with rough mutant *Escherichia coli*. *J. Infect. Dis.* **163**:769-772.
- Bhutta, Z., B. J. Farooqui, and A. W. Sturm. 1992. Eradication of a multiple drug resistant *Salmonella paratyphi* A causing meningitis with ciprofloxacin. *J. Infect.* **25**:215-219.
- Bock, K., M. Meldal, D. R. Bundle, T. Iversen, P. J. Garegg, T. Norberg, A. A. Lindberg, and S. B. Svenson. 1984. The conformation of *Salmonella* O-antigenic polysaccharide chains of serogroups A, B, and D<sub>1</sub> predicted by semi-empirical hard-sphere (HSEA) calculations. *Carbohydr. Res.* **130**:23-34.
- Bock, K., M. Meldal, D. R. Bundle, T. Iversen, B. Mario Pinto, P. J. Garegg, I. Kvanstrom, T. Norberg, A. A. Lindberg, and S. B. Svenson. 1984. The conformation of *Salmonella* O-antigenic oligosaccharides of serogroup A, B, and D<sub>1</sub> inferred from <sup>1</sup>H- and <sup>13</sup>C-nuclear magnetic resonance spectroscopy. *Carbohydr. Res.* **130**:35-53.

10. Carlin, N. I. A., S. B. Svenson, and A. A. Lindberg. 1987. Role of monoclonal O-antigen antibody epitope specificity and isotype in protection against experimental mouse typhoid. *Microb. Pathog.* **2**:171-183.
11. Carter, P. B., and F. M. Collins. 1974. Growth of typhoid and paratyphoid bacilli in intravenously infected mice. *Infect. Immun.* **10**:816-822.
12. Chalker, R. B., and M. J. Blaser. 1988. A review of human salmonellosis. III. Magnitude of *Salmonella* infection in the United States. *Rev. Infect. Dis.* **10**:111-124.
13. Chu, C., B. Liu, D. Watson, S. C. Szu, D. Bryla, J. Shiloach, R. Schneerson, and J. B. Robbins. 1991. Preparation, characterization, and immunogenicity of conjugates composed of the O-specific polysaccharide of *Shigella dysenteriae* type 1 bound to tetanus toxoid. *Infect. Immun.* **59**:4450-4458.
14. Claesson, B. O., B. Trollfors, T. Lagergard, J. Taranger, D. Bryla, G. Oterman, T. Cramton, Y. Yang, C. B. Reimer, J. B. Robbins, and R. Schneerson. 1988. Clinical and immunologic responses to the capsular polysaccharide of *Haemophilus influenzae* type b alone or conjugated to tetanus toxoid in 18- to 23-month-old children. *J. Pediatr.* **112**:695-702.
15. Cohen, D., S. Ashkenazi, M. S. Green, M. Gdalevich, G. Robin, R. Slepion, M. Yavzori, N. Orr, C. Block, I. Ashkenazi, J. Shemer, D. N. Taylor, T. L. Hale, J. C. Sadoff, D. Pavliakova, R. Schneerson, and J. B. Robbins. 1997. Double-blind vaccine-controlled randomized efficacy trial of an investigational *Shigella sonnei* conjugate vaccine in young adults. *Lancet* **349**:155-159.
16. Colwell, D. E., S. M. Michalek, D. E. Briles, J. Emilio, and J. R. McGhee. 1984. Monoclonal antibodies to *Salmonella* lipopolysaccharide: anti-O-polysaccharide antibodies protect C3H mice against challenge with virulent *Salmonella typhimurium*. *J. Immunol.* **133**:950-957.
17. Cygler, M., D. R. Rose, and D. R. Bundle. 1991. Recognition of a cell-surface oligosaccharide of pathogenic *Salmonella* by an antibody Fab fragment. *Science* **253**:442-445.
18. DeMaria, A., M. A. Johns, H. Berberich, and W. R. McCabe. 1988. Immunization with rough mutants of *Salmonella minnesota*: initial studies in human subjects. *J. Infect. Dis.* **158**:301-311.
19. Ebong, W. W. 1986. Acute osteomyelitis in Nigerians with sickle cell disease. *Ann. Rheum. Dis.* **45**:911-915.
20. Edwards, P. R., and D. W. Bruner. 1943. The occurrence and distribution of *Salmonella* types in the United States. *J. Infect. Dis.* **72**:58-61.
21. Edwards, P. R., and W. H. Ewing. 1972. Identification of Enterobacteriaceae, 3rd ed., p. 175. Burgess Publishing Company, Minneapolis, Minn.
22. Escamilla, J., H. Florez-Ugarte, and M. E. Kilpatrick. 1986. Evaluation of blood clot cultures for isolation of *Salmonella typhi*, *Salmonella paratyphi A*, and *Brucella melitensis*. *J. Clin. Microbiol.* **24**:388-390.
23. Fouquey, C., J. Polonsky, E. Lederer, O. Westphal, and O. Luderitz. 1958. Synthesis of 3,6-dideoxy-D-ribo-hexose (3,6-dideoxy-D-glucose) and its identification with paratose. *Nature* **182**:944.
24. Gerichter, C. B. 1960. The dissemination of *Salmonella typhi*, *Salmonella paratyphi A*, and *Salmonella paratyphi B* through the organs of the white mouse by oral infection. *J. Hyg. Camb.* **58**:307-319.
25. Hafiz, S., S. W. Khan, R. Shariff, I. Yazdani, Y. Syed, and T. Hafiz. 1993. Epidemiology of salmonellosis and its sensitivity in Karachi. *J. Pak. Med. Assoc.* **43**:178-179.
26. Hargrett-Bean, N. T., A. T. Pavia, and R. V. Tauxe. 1988. *Salmonella* isolates from humans in the United States, 1984-1986. *Morbidity and Mortality Weekly Report* **37**(SS-2):25-31.
27. Hellerqvist, C. G., B. Lindberg, K. Samuelsson, and A. A. Lindberg. 1971. Structural studies on the O-specific side-chains of the cell-wall lipopolysaccharide from *Salmonella paratyphi A* var. durazzo. *Acta Chem. Scand.* **25**:955-961.
28. Hien, T. T., D. B. Bethell, N. T. T. Hoa, J. Wain, T. S. Diep, L. T. Phi, B. M. Cuong, N. M. Duong, P. T. Thanh, A. L. Walsh, N. P. J. Day, and N. J. White. 1995. Short course of ofloxacin for treatment of multidrug-resistant typhoid. *Clin. Infect. Dis.* **20**:915-923.
29. Jude, A., and L. Le Minor. 1948. *Salmonella* isolées en Indochine au cours d'affections typho-paratyphoidiques. *Bull. Soc. Pathol. Exot.* **41**:129-133.
30. Kabat, E. A., and A. E. Bezer. 1958. The effect of variation in molecular weight on the antigenicity of dextran in man. *Arch. Biochem. Biophys.* **78**:306-310.
31. Kapil, A., A. Sood, V. P. Reddaiah, B. Das, and P. Seth. 1997. Paratyphoid fever due to *Salmonella enterica* serotype paratyphi A. *Emerg. Infect. Dis.* **3**:407.
32. Kauffmann, F. 1954. Enterobacteriaceae, 2nd ed., p. 107. Munksgaard, Copenhagen, Denmark.
33. Kohn, J., and M. Wilchek. 1983. 1-Cyano-4-dimethylamino pyridinium tetrafluoroborate as a cyanylating agent for the covalent attachment of ligand to polysaccharide resins. *FEBS Lett.* **154**:209-210.
34. Konadu, E., J. B. Robbins, J. Shiloach, D. A. Bryla, and S. C. Szu. 1994. Preparation, characterization, and immunological properties in mice of *Escherichia coli* O157 O-specific polysaccharide-protein conjugate vaccines. *Infect. Immun.* **62**:5048-5054.
35. Konadu, E., J. Shiloach, D. A. Bryla, J. B. Robbins, J. B., and S. C. Szu. 1996. Synthesis, characterization, and immunological properties in mice of conjugates composed of detoxified lipopolysaccharide of *Salmonella paratyphi A* bound to tetanus toxoid, with emphasis on the role of O-acetyls. *Infect. Immun.* **64**:2709-2715.
36. Lasserre, R., R. P. Sangalang, and L. Santiago. 1991. Three-day treatment of typhoid fever with two different doses of ceftriaxone, compared to 14-day therapy with chloramphenicol: a randomized trial. *J. Antimicrob. Chemother.* **28**:765-772.
37. Lee, S.-C., P.-H. Yang, W.-B. Shieh, and R. Lasserre. 1994. Bacteremia due to non-typhi *Salmonella*: analysis of 64 cases and review. *Clin. Infect. Dis.* **19**:693-696.
38. Lees, A., B. L. Nelson, and J. J. Mond. 1996. Activation of soluble polysaccharides with 1-cyano-4-dimethylaminopyridium tetrafluoroborate for use in protein-polysaccharide conjugate vaccines and immunological reagents. *Vaccine* **14**:190-198.
39. Lepage, P., J. Bogaerts, C. van Goethem, M. Ntahorutaba, F. Nsengumuremyi, D. G. Hitimana, J. Vandepitte, J.-P. Butzler, and J. Levy. 1987. Community-acquired bacteremia in African children. *Lancet* **i**:1458-1461.
40. Leung, M. 1995. Simultaneous infection by a sensitive and a multiresistant strain of *Salmonella paratyphi A*. *J. Infect.* **30**:181-183.
41. Mahanta, J. 1994. Drug sensitivity of *Salmonella paratyphi A* isolated from a suspected outbreak of fever in Dulijan. *J. Indian Med. Assoc.* **92**:49-50.
42. Nesbitt, A., and N. B. Mirza. 1989. *Salmonella* septicaemias in Kenyan children. *J. Trop. Pediatr.* **35**:35-39.
43. Nguyen, T. A., H. K. Ha, and T. D. Nguyen. 1993. Typhoid fever in South Vietnam, 1990-1993. *Bull. Soc. Pathol. Exot.* **86**:476-478.
44. Nourmand, A., M. Ziai, and A. C. Tahernia. 1971. Typhoid-paratyphoid fevers in infancy. *Clin. Pediatr.* **10**:272-274.
45. Ørskov, F., and I. Ørskov. 1983. Summary of a workshop on the clone concept in the epidemiology, taxonomy, and evolution of the *Enterobacteriaceae* and other bacteria. *J. Infect. Dis.* **148**:346-357.
46. Phillips, I., and B. Wharton. 1968. Acute bacterial infection in kwashiorkor and marasmus. *Br. Med. J.* **1**:407-409.
47. Rao, P. S., I. Bairy, P. V. Rao, and P. G. Shivananda. 1994. Multiresistant *Salmonella paratyphi A* infection in coastal Karnataka. *J. Assoc. Physicians India* **42**:929.
48. Raynal, J. H., and J. Fournier. 1947. Les salmonelles à Changhaï. *Med. Trop.* **7**:199-237.
49. Reeves, M. W., G. M. Evins, A. A. Heiba, B. D. Plikaytis, and J. J. Farmer III. 1989. Clonal nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis and proposal of *Salmonella bongori* comb. nov. *J. Clin. Microbiol.* **27**:313-320.
50. Robbins, J. B., C. Y. Chu, and R. Schneerson. 1992. Hypothesis for vaccine development: protective immunity to enteric diseases caused by nontyphoidal salmonellae and shigellae may be conferred by serum IgG antibodies to the O-specific polysaccharide of their lipopolysaccharides. *Clin. Infect. Dis.* **15**:346-361.
51. Robbins, J. B., and R. Schneerson. 1990. Polysaccharide-protein conjugates: a new generation of vaccines. *J. Infect. Dis.* **161**:821-832.
52. Robbins, J. B., R. Schneerson, and S. C. Szu. 1995. Perspective: hypothesis: serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating the inoculum. *J. Infect. Dis.* **171**:1387-1398.
53. Rowe, B., and R. J. Gross. 1984. Genus II. *Salmonella*, p. 423-458. In N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore, Md.
54. Sarnaik, S., J. Kaplan, G. Schiffman, D. A. Bryla, J. B. Robbins, and R. Schneerson. 1990. Studies on pneumococcus vaccine alone or mixed with DTP and on pneumococcus type 6B and *Haemophilus influenzae* type b capsular polysaccharide-tetanus toxoid conjugates in 2- to 5-year-old children with sickle cell anemia. *Pediatr. Infect. Dis.* **9**:181-186.
55. Schwartz, E., D. R. Schlim, M. Eaton, N. Jenks, and R. Houston. 1988. The effect of oral and parenteral typhoid vaccination on the rate of infection with *Salmonella typhi* and *Salmonella paratyphi A* among foreigners in Nepal. *Arch. Intern. Med.* **150**:349-351.
56. Selander, R. K., P. Beltran, N. H. Smith, R. Helmuth, F. A. Rubin, D. J. Kopecko, K. Ferris, B. D. Tall, A. Cravioto, and J. M. Musser. 1990. Evolutionary genetic relationships of clones of *Salmonella* serovars than cause human typhoid and other enteric fevers. *Infect. Immun.* **58**:2262-2275.
57. Simanjuntak, C. H., F. P. Paleologo, N. H. Punjabi, R. Darmowigoto, H. Totosudirjo, P. Haryanto, E. Suprijanto, N. D. Witham, and S. L. Hoffman. 1991. Oral immunization against typhoid fever in Indonesia with Ty21a vaccine. *Lancet* **338**:1055-1059.
58. Svensson, S. B., and A. A. Lindberg. 1981. Artificial *Salmonella* vaccines: *Salmonella typhimurium* O-antigen-specific oligosaccharide-protein conjugates elicit protective antibodies in rabbits and mice. *Infect. Immun.* **32**:490-496.
59. Syverton, J. T., R. E. Ching, and A. B. Smith. 1946. Typhoid and paratyphoid A in immunized military personnel. *JAMA* **131**:507-514.
60. Szu, S. C., X. Li, R. Schneerson, J. H. Vickers, D. Bryla, and J. B. Robbins. 1989. Comparative immunogenicities of Vi polysaccharide-protein conjugates composed of cholera toxin or its B subunit as a carrier bound to high- or lower-molecular-weight Vi. *Infect. Immun.* **57**:3823-3827.
61. Szu, S. C., R. Gupta, and J. B. Robbins. 1994. Induction of serum vibriocidal antibodies by O-specific polysaccharide-protein conjugate vaccines for preven-

- tion of cholera, p. 381–394. In I. K. Wachsmuth, P. A. Blake, and O. Olsvik (ed.), *Vibrio cholerae*. American Society for Microbiology, Washington, D.C.
62. **Thisyakorn, U., P. Mansuwan, and D. N. Taylor.** 1987. Typhoid and paratyphoid fever in 192 hospitalized children in Thailand. *Am. J. Dis. Child.* **141**:862–865.
  63. **Vergese, S. L., R. Manonmani, S. Balasubramanian, and S. Chandrasekharan.** 1992. Multi-drug resistance in salmonellae isolated from enteric fever cases at Porur—a semi urban area near Madras City. *J. Commun. Dis.* **24**:12–15.
  64. **Watson, D. C., J. B. Robbins, and S. C. Szu.** 1992. Protection of mice against *Salmonella typhimurium* with an O-specific polysaccharide-protein conjugate vaccine. *Infect. Immun.* **60**:4679–4686.
  65. **World Health Organization Expert Committee on Biologic Standardization.** 1993. Requirements on Vi polysaccharide for typhoid. W. H. O. Tech. Rep. Ser. **43**:14–32.
  66. **Zimmerman, L. E., M. Cooper, and C. D. Graber.** 1952. Bacteriologic studies in an outbreak of salmonellosis in Korea. *Am. J. Hyg.* **56**:252–264.

---

*Editor:* J. T. Barbieri