JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1993, p. 975–978
0095-1137/93/040975-04$02.00/0

Vol. 31, No. 4

Salmonella typhi O:9,12 Polysaccharide-Protein Conjugates: Characterization and Immunoreactivity with Pooled and Individual Normal Human Sera, Sera from Patients with Paratyphoid A and B and Typhoid Fever, and Animal Sera

LIESELOTTE ARON,[1] JOSE DI FABIO,[2]† AND FELIPE C. CABELLO[1]*

Department of Microbiology and Immunology, New York Medical College, Valhalla, New York 10595,[1] and Division of Biological Sciences, National Research Council Canada, Ottawa, Ontario K1A OR6, Canada[2]

Received 3 February 1992/Accepted 21 December 1992

Polysaccharide of O:9,12 specificity purified from *Salmonella typhi* was conjugated to tetanus toxoid or bovine serum albumin in order to obtain defined antigenic material that would contain O chain free of other *S. typhi* antigens and that would be suitable for characterizing host humoral response to only *S. typhi* O-chain antigens. These artificial conjugates were strongly reactive in immunodots with 18 pooled and 3 individual serum samples from patients with typhoid fever and with rabbit anti-*Salmonella* O antiserum (group D, factors 1, 9, and 12). They reacted weakly with one serum sample from one human with paratyphoid A. These results suggest that the periodate oxidation and the reductive amination used in the conjugation conserved the immunogenicity of the O chain and allowed its absorption to nitrocellulose. They also suggest that the bovine serum albumin conjugate could be used in the diagnosis of *S. typhi* infections as normal sera may react with the protein molecule of the tetanus toxoid conjugate.

Study of the human response to gram-negative bacteria has been hindered both by their complex surface structure (2, 3, 21) and by an inability to purify each individual component completely (2, 11, 21). Interpretation of host humoral response against *Salmonella* antigens in Western blots (immunoblots) has been complicated both by the presence of denatured protein(s) and antigenic preparations contaminated with lipopolysaccharide (LPS) and by the presence of anti-LPS antibodies in patients' and developing sera (2, 3, 6, 7, 12). Diagnostic serological assays for typhoid fever and *Salmonella typhi* carriers based on immune response to *S. typhi* O, H, and Vi antigens lack specificity and sensitivity, in part because of their use of unpurified antigens (11). To facilitate the study of human immune response to *S. typhi* O antigen, *S. typhi* O antigen was conjugated to bovine serum albumin (BSA) or tetanus toxoid (TT) and reactivity with various human and animal anti-*Salmonella* antibodies was analyzed by Western and immunodot blots.

Preparation of O:9,12 conjugates. *S. typhi* LPS (Sigma, St. Louis, Mo.) was purified (8, 16), separated from free lipid A by electrodialysis (5, 16), selectively oxidized (1, 9, 13, 15, 19), and conjugated (15, 19) to either TT (Bureau of Biologics, Health and Welfare Canada, Ottawa, Ontario) or BSA (Sigma). Conjugated material was dialyzed against distilled water and lyophilized. Protein in the conjugates was quantitated by using BSA as a standard (18). Carbohydrate in the conjugates was quantitated by using purified *S. typhi* O chain as a standard (9, 10). Galactose was used in standardization and validation of the phenol-sulfuric acid test (9, 10). TT conjugates 1 and 2 were 40 and 23% carbohydrate and 60 and 77% protein, respectively. A single BSA conjugate was 70% protein and 30% carbohydrate. The 100% total refers solely to the carbohydrate and protein content. Usually, our glycoconjugates correspond to between 75 and 90% of the total weight, depending on the number of dialysis steps. Assuming a molecular mass of 15 kDa for the polysaccharide, an average of 7 and 3 O chains per TT molecule were linked for conjugates 1 and 2, respectively, and an average of 10 O chains per BSA molecule were linked. Conjugations have been repeated at least two more times, generating conjugates of similar composition.

Sera and antibodies. Sera were obtained from patients with bacteriologically proved typhoid fever during the first week of disease during the summer of 1987 in Santiago, Chile. (one serum pool from 18 patients, three unpooled individual serum samples). Sera from two paratyphoid A and two paratyphoid B patients, whose infections were confirmed by blood culture, were obtained during the first week of disease during the summer of 1990 in Santiago, Chile. A serum pool from six healthy volunteers and two individual serum samples from individuals in a nonendemic area with no known contact with *S. typhi* were used as controls. Antibodies were semipurified from heat-inactivated sera (30 min at 56°C) by three rounds of $(NH_4)_2SO_4$ precipitation (14), and the anti-*S. typhi* LPS component of an aliquot was removed by absorption (see below). When semipurified immunoglobulins were used, their concentrations were adjusted to that present in the original sera. Rabbit anti-*Salmonella* O antiserum (group D, factors 1, 9, and 12) was obtained from Difco. Guinea pig anti-TT serum was obtained from the Division of Bacterial Products, Bureau of Biologics, and heat inactivated as described above. Dot immunoblots were used to detect antibodies against TT, BSA, and smooth *S. typhi* LPS. All human sera contained antibodies against TT. TT antibodies were absorbed with nitrocellulose sheets moistened with 0.5 mg of TT (1:100) in Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 8.5; 250 mM NaCl) blocked in 5% BSA–5% skim milk–TBS for 1 h at 37°C. Antibody dilutions were incubated with strips of TT-nitrocellulose until no more anti-TT activity was detected by immunodot assay. Anti-*S.*

* Corresponding author.
† Present address: Bureau of Biologics, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario K1A OL2, Canada.

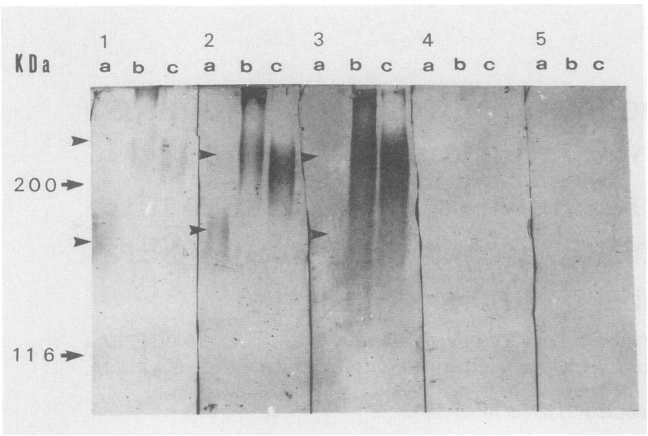

FIG. 1. Western blot of tetanus toxoid and its *S. typhi* O-chain polysaccharide conjugates. Lanes a, tetanus toxoid; lanes b, conjugate 1; lanes c, conjugate 2. Section 1, India ink stain (large arrows indicate molecular masses); section 2, reaction with guinea pig anti-TT serum; section 3, reaction with the typhoid fever convalescent-phase serum immunoglobulin pool depleted of anti-TT antibodies; section 4, reaction with the typhoid fever convalescent-phase serum immunoglobulin pool depleted of anti-TT and anti-*S. typhi* LPS antibodies; section 5, reaction with the normal human immunoglobulin pool depleted of anti-TT antibodies. Blocking and dilutions were done in 5% skim milk–5% BSA–TBS. The upper small arrows mark the position of migration of the conjugates. The lower small arrows mark the position of migration of the TT.

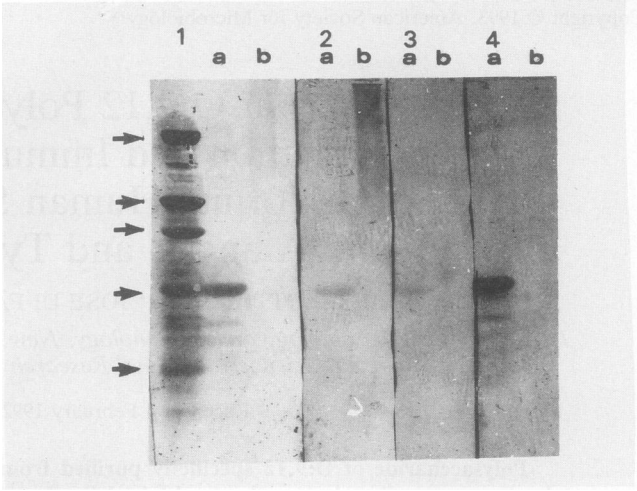

FIG. 2. Western blot of BSA (lanes a) and its *S. typhi* O-chain polysaccharide conjugate (lanes b). Blocking and serum dilutions were done in 0.3% Tween 20–1% skim milk–TBS. Section 1, India ink stain (the left lane contains molecular mass markers, indicated by arrows); section 2, reaction with the typhoid fever convalescent-phase serum antibody pool; section 3, same as section 2 but depleted of anti-*S. typhi* LPS activity; section 4, reaction with the normal human antibody pool. Molecular mass markers (from top to bottom): 200, 116, 97.4, 66.2, and 42.7 kDa.

typhi LPS antibodies were removed either by passage over a column of LPS-AH Sepharose at 4°C prepared with 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC; Sigma) as a coupling agent (3) or by absorption with nitrocellulose sheets saturated with purified *S. typhi* LPS (about 0.5 mg for each sheet [10 by 10 cm]), *Salmonella typhimurium* TV119 R_a LPS, or *Salmonella minnesota* 595 R_e LPS (3).

Western blotting and immunodot assays. TT (2 μg), two TT–O-antigen conjugates (4 μg of protein each), BSA, and the BSA conjugate were electrophoresed on a 7% polyacrylamide–sodium dodecyl sulfate (SDS) gel (17) and transferred to nitrocellulose (Bio-Rad) in a Polyblot cell (model SBD-1000; American Bionetics) according to the manufacturer's instructions. Immunodot assays on nitrocellulose sheets for serial dilutions of native BSA (200 to 12.5 ng) and its O-antigen conjugate (200 to 12.5 ng of protein) were done with a blotting cell (Bio-Dot; Bio-Rad).

Western blot immunoanalysis of TT, BSA, and their O-antigen conjugates. TT, BSA, and O-antigen conjugates separated in SDS-polyacrylamide gel electrophoresis (PAGE) gels were analyzed by Western blot (Fig. 1 and 2). Both TT conjugates and the BSA conjugate reacted with convalescent-phase sera from typhoid fever patients when tested on Western blots and by immunodot assays. The Western blot results presented in Fig. 1, lanes b and c, and Fig. 2, lanes b, also indicated that the O antigen was covalently linked to protein, because after conjugation the protein band disappeared in SDS-PAGE and a new, high-molecular-weight band contained both protein and O antigen. The TT conjugate was inconvenient to use, as it required preabsorption of anti-TT antibodies from both human and animal sera. After removal of these antibodies by a single-step absorption, only typhoid fever convalescent-phase sera exhibited reaction with the TT O-chain polysaccharide conjugates (Fig. 1, section 3). Removal of anti-*S. typhi* LPS antibodies by absorption with *S. typhi* LPS completely abolished reactivity with both conjugates (Fig. 1, section 4). Immune reactivity of convalescent-phase sera with *S. typhi* LPS was completely abolished by combined absorption with these conjugates and rough *S. typhimurium* LPS. This indicates that most, if not all, of the immunoreactivity of *S. typhi* O antigen was preserved in the TT conjugates and that the degree of substitution did not abolish their reactivity with a specific anti-TT serum.

Immunodot assays. All convalescent-phase sera exhibited positive reactions with the BSA–O-antigen conjugate at conjugate doses up to 12.5 ng; the anti-*S. typhi* LPS-depleted pool did not react (Fig. 3).

Commercial anti-*S. typhi* LPS rabbit serum had consistently low titers with BSA–O-antigen conjugate. None of the sera reacted with native BSA. Normal human antibody pool serum, two normal human serum samples, nonimmune rabbit serum, and guinea pig anti-TT serum did not react with either native unconjugated or O-antigen-conjugated BSA. Only one paratyphoid A serum sample showed reaction with the conjugate, albeit more faintly than the typhoid fever serum and only at doses of 25 ng. Again, no reaction against native unconjugated BSA was apparent.

We have generated synthetic and well-characterized protein conjugates of *S. typhi* O-chain polysaccharide that are free of any other *S. typhi* antigens (including cross-reacting rough polysaccharide) and that are specifically recognized by antibodies present in the sera of patients with typhoid fever. The coupling method used minimizes polysaccharide and protein modification, preserves immunoreactivity and probably immunogenicity, avoids protein cross-linking (1), prevents the formation of a potentially carcinogenic aromatic amine (1, 15, 19), and allows the absorption of *S. typhi* O antigen to nitrocellulose. The BSA conjugate was considerably more useful than the tetanus conjugate. Although most human and animal sera reacted with SDS-denatured BSA in the presence of Tween 20, they showed no reaction with native BSA. When Western blots of BSA and its

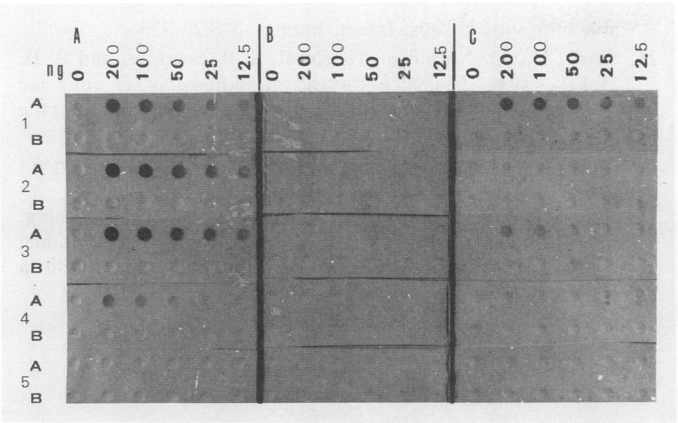

FIG. 3. Immunodot assay for BSA and its O-antigen conjugates. Blocking, serum dilutions, and washings were performed in 0.3% Tween–1% skim milk–TBS. Rows A and B contain serial dilutions of undenatured BSA–O-antigen conjugate and BSA, respectively, with the amount of antigen indicated at the top (in nanograms of protein. (Panel A) All sections reacted with immune sera, as follows: the typhoid fever convalescent-phase antibody pool (section 1), two typhoid fever convalescent-phase sera obtained immediately after confirmation of the disease (sections 2 and 3), rabbit anti-*S. typhi* anti-O-antigen antiserum (section 4), and the anti-O-antigen-depleted typhoid fever antibody pool (section 5). (Panel B) All sections reacted with nonimmune (no known history of *Salmonella* infection) sera, as follows: the normal antibody pool (section 1), two individual normal serum samples (sections 2 and 3), rabbit serum (section 4), and guinea pig anti-TT serum (section 5). (Panel C) All sections reacted with immune sera, as follows: another typhoid fever convalescent-phase serum (section 1), two paratyphoid A patient serum samples (sections 2 and 3), and two paratyphoid B patient serum samples (sections 4 and 5).

conjugate were reacted with sera in the presence of 5% skim milk–5% BSA, only convalescent-phase sera from typhoid fever patients and rabbit anti-*S. typhi* O-antigen serum reacted with the conjugate (Fig. 3). This reaction was specific since removal of the anti-*S. typhi* LPS antibodies from convalescent-phase sera by absorption with *S. typhi* LPS completely abolished reaction with the conjugate (Fig. 2, section 3). On the other hand, all sera processed in the presence of Tween 20 reacted with denatured unconjugated BSA, but only convalescent-phase serum immunoglobulins from typhoid fever patients reacted with the conjugate. Absorption of anti-*S. typhi* LPS antibodies eliminated this latter reaction completely, confirming its specificity. This indicated that a high degree of substitution of BSA with O-chain polysaccharide blocked any potential immunoreactive groups on the denatured BSA molecule in a manner reminiscent of the blocking of porin protein immunoreactivity by O polysaccharide (2, 4, 23). This interpretation is also supported by the poor staining of this conjugate with India ink.

Of the four paratyphoid patient sera, only one reacted weakly with the *S. typhi* O-antigen–BSA conjugate at the highest concentration tested (Fig. 3, section 3C). Since the group D *Salmonella* O-antigen specificity resides in the O:9 antigen (15, 19, 21) and since the O:12 antigen associated with the mannose-rhamnose-galactose backbone of the O polysaccharide of this group is also shared by other salmonellae (20), a more specific O:9-antigen diagnostic tool for typhoid fever would be a 3-*O*-(α-tyvelopyranosyl)-α-D-mannopyranoside–BSA conjugate, already used as a diagnostic tool in rabbits (22). The facts that two convalescent-phase sera were obtained immediately after confirmation of typhoid fever, coupled with the absence of reaction of all sera tested with the BSA carrier (both native and SDS and heat denatured), and that only one paratyphoid patient serum sample gave a weak reaction increase the potential feasibility of using this conjugate as a diagnostic reagent.

This work was supported by grant RO1 AI33613 from the National Institute of Allergy and Infectious Diseases and from the American Heart Association and Eastern Paralyzed Veterans Association to F. C. Cabello.

The sera from typhoid fever patients were provided to us by E. Zunino and M. A. Castrillon, Santiago, Chile. We thank Henry Godfrey and Harriett V. Harrison for their help in the preparation of the manuscript.

REFERENCES

1. **Aalmo, K. M., M. F. Isahk, and T. Painter.** 1978. Possibilities for selective Smith-degradation. Carbohydr. Res. **63:**C3–C7.
2. **Aguero, J., G. Mora, M. J. Mroczenski-Widley, M. E. Fernandez-Beros, L. Aron, and F. C. Cabello.** 1987. Cloning, expression and characterization of 36 kdal *Salmonella typhi* porin gene in *Escherichia coli*. Microb. Pathog. **3:**399–407.
3. **Aron, L., G. Faundez, C. Gonzalez, E. Roessler, and F. C. Cabello.** 1993. Lipopolysaccharide-independent radioimmunoprecipitation and identification of structural and *in vivo* induced immunogenic surface proteins of *Salmonella typhi* in typhoid fever. Vaccine **11:**10–17.
4. **Bentley, A. T., and P. E. Klebba.** 1988. Effect of lipopolysaccharide structure on reactivity of antiporin monoclonal antibodies with the bacterial cell surface. J. Bacteriol. **170:**1063–1068.
5. **Bligh, E. J., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
6. **Brown, A., and C. E. Hormache.** 1989. The antibody response to salmonellae in mice and humans: studies by immunoblots and ELISA. Microb. Pathog. **6:**445–454.
7. **Calderón, I., S. R. Lobos, H. A. Rojas, C. Palomino, L. H. Rodríguez, and G. C. Mora.** 1986. Antibodies to porin antigens of *Salmonella typhi* induced during typhoid infection in humans. Infect. Immun. **52:**209–212.
8. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155:**831–838.
9. **Di Fabio, J. L., M. B. Perry, and J. R. Brisson.** 1988. Structure of the antigenic O-polysaccharide of the lipopolysaccharide produced by *Salmonella eimsbuttel.* Biochem. Cell. Biol. **66:** 107–115.
10. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Revers, and F. Smith.** 1956. Colorimetric method for the determination of sugars and related substances. Anal. Chem. **28:**350–356.
11. **Edelman, R., and M. M. Levine.** 1986. Summary of an international workshop on typhoid fever. Rev. Infect. Dis. **8:**329–349.
12. **Fernandez-Beros, M. E., C. Gonzalez, M. A. McIntosh, and F. C. Cabello.** 1989. Immune response to the iron-deprivation-induced proteins of *Salmonella typhi* in typhoid fever. Infect. Immun. **57:**1271–1275.
13. **Goldstein, I. J., G. W. Hay, B. A. Lewis, and F. Smith.** 1965. Controlled degradation of polysaccharides by periodate oxidation, reduction and hydrolysis. Methods Carbohydr. Chem. **5:**361–370.
14. **Hudson, L. E., and F. C. Hay.** 1980. Practical immunology, p. 156–177. Blackwell Scientific Publications, Oxford.
15. **Jennings, H. J., and C. Lugowski.** 1981. Immunochemistry of groups A, B and C meningococcal polysaccharide-tetanus toxoid conjugates. J. Immunol. **127:**1011–1018.
16. **Karkhanis, Y. D., J. Y. Zeltner, J. J. Jackson, and D. J. Carlo.** 1978. A new and improved micro-assay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram-negative bacteria. Anal. Biochem. **85:**595–601.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the

assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

18. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1961. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
19. **Lugowski, C., M. Kulakowska, and E. Romanowska.** 1986. Characterization and diagnostic application of a lipopolysaccharide core oligosaccharide-protein conjugate. J. Immunol. Methods **95**:187–194.
20. **Robertsson, J. A., C. Fossum, S. B. Svenson, and A. A. Lindberg.** 1982. *Salmonella typhimurium* infection in calves: specific immune reactivity against O-antigenic polysaccharide detectable in in vitro assays. Infect. Immun. **37**:728–736.
21. **Saxen, H., M. Nurminen, N. Kuusi, S. B. Soerison, and P. H. Makela.** 1986. Evidence for the importance of O antigenic antibodies in mouse-protective *Salmonella* outer membrane (porin) antisera. Microb. Pathog. **1**:433–441.
22. **Svenson, S. B., and A. A. Lindberg.** 1983. Artificial *Salmonella* vaccines. Prog. Allergy **33**:120–143.
23. **van der Ley, P., O. Kuipers, J. Tommassen, and B. Lugtenberg.** 1986. O-antigenic chains of lipopolysaccharide prevent binding of antibody molecules to an outer membrane pore protein in Enterobacteriaceae. Microb. Pathog. **1**:43–49.