Characterization of Monoclonal Antibodies to Protein Antigen of Salmonella typhi

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Two monoclonal antibodies were produced against protein antigens of Salmonella typhi. One of the antibodies (STP14) belongs to the immunoglobulin G1K subclass, and the other (STP13) was assigned to the immunoglobulin G2a(κ) subclass. Both antibodies could recognize the 34.0-kilodalton protein antigen from S. typhi. The specificity of these antibodies was tested by immunoblotting with a panel of crude protein antigens from 12 bacteria causing enteric fever and enteric fever-like illness: S. typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C, S. choleraesuis, S. enteritidis, S. krefeld, S. panama, S. typhimurium, Escherichia coli, Pseudomonas pseudomallei, and Yersinia enterocolitica. In a modified double-antibody sandwich enzyme-linked immunosorbent assay they could detect the protein antigen at ca. 0.6 μg/ml. These monoclonal antibodies should be of great value in the diagnostic test for detecting S. typhi antigen in samples of bodily fluids isolated from patients with typhoid fever and in studies on the chemical structure and other immunological properties of this 34.0-kilodalton protein.

One of the problems of diagnosis of typhoid fever is that of differentiating it from typhoid fever-like illness caused by members of the family Enterobacteriaceae other than Salmonella typhi. The most dependable way to establish a definitive diagnosis is to use hemoculture. However, laboratory diagnosis by hemoculture has certain disadvantages. Many cultures are falsely negative owing to prior antibiotic therapy. Even when appropriate hemocultures are taken, the presumptive bacteriological results require at least 48 h for confirmative results.

Serological diagnosis of typhoid fever by the Widal test has been found by some investigators to be unreliable (1, 11), and confirmation requires both acute- and convalescent-phase serum samples, which are rarely collected (11). Hence, the antibodies in single serum specimen are not very useful for serodiagnosis, since they cannot distinguish current from previous infection.

Therefore, there is a need for a test that permits rapid laboratory diagnosis of typhoid fever by using a single specimen. That test should be directed toward detecting S. typhi antigens from the bodily fluids of patients. Many investigators have established such tests by using conventional polyclonal antisera, but they have had problems with cross-reactions of antisera with other members of the family Enterobacteriaceae (2, 6, 8, 13, 14). Attempts to reduce the cross-reactivities by absorption have not been able to eliminate all of the cross-reactive antibodies (8). To overcome this problem, monoclonal antibodies (MAbs) which are specific to antigens of S. typhi are required. In this paper we report the establishment of two MAbs specific to the 34.0-kilodalton (kDa) protein antigen of S. typhi.

**MATERIALS AND METHODS**

**Bacteria.** All bacteria used in this study can cause enteric fever and enteric fever-like illness: S. typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C, S. choleraesuis, S. enteritidis, S. krefeld, S. panama, S. typhimurium, Escherichia coli, Pseudomonas pseudomallei, and Yersinia enterocolitica.

**Preparation of protein antigens.** The protein (Bp) antigens from all bacteria were prepared by the method originally described by Barber et al. (4). Briefly, washed and acetone-dried bacterial cells were extracted with Veronal buffer (pH 8.4), and the protein was precipitated out of the Veronal buffer extract with trichloroacetic acid. The precipitate was washed, dissolved, lyophilized, and stored at 4°C until used. The protein content was assayed by the method described by Lowry et al. as modified by Hartree (7). This antigen had a small amount of lipopolysaccharide contamination as confirmed by double immunodiffusion.

**Production of MAbs.** Two female BALB/cj mice 6 to 10 weeks of age (kindly provided by the Division of Veterinary Medicine, Armed Force Research Institute of Medical Science, Bangkok, Thailand) were immunized once every 3 days for 15 days (9). For the first immunization, each mouse was given 200 μg of S. typhi Bp (Bp-ST), diluted to 0.25 ml in phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund adjuvant (Difco Laboratories, Detroit, Mich.), which was injected subcutaneously into two footpads and two axillary and two inguinal areas. The second immunization was similar to the first, except that incomplete Freund adjuvant was used. For immunizations 3 to 6, each mouse was given 200 μg of Bp-ST, in PBS without adjuvant, which was injected subcutaneously into the six areas mentioned above, except that for immunization 6, the antigen was divided and half was given intravenously for spleen collection. At 7 days after the last administration of antigen, mice were sacrificed by cervical dislocation. The axillary and inguinal lymph nodes and one spleen were aseptically removed for hybridization. Two separate fusions were performed: 7.8 × 10^7 spleen cells were fused with 4 × 10^7 P3x63-Ag8.653 mouse myeloma cells, and 3.5 × 10^6 lymph node cells were fused with 3.5 × 10^6 P3X63-Ag8.653 cells by using 42% polyethylene glycol 4000 (Accurate Chemical and Scientific Co., Westbury, N.Y.). The fused cells were dispensed into 24-well tissue culture plates (Costar, Cambridge, Mass.) in the presence of syngeneic mouse peritoneal feeder cells and cultured at 37°C at 80% humidity under 7% CO2. Hybridomas secreting specific antibodies were cloned by the single-
dilution method as follows. Antibody-positive hybridoma cells (1 to 5 μl) were transferred to 10 ml of complete medium (400 ml of RPMI 1640, 20% fetal bovine serum, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml) in the presence of syngeneic mouse peritoneal feeder cells. A 5-ml pipette was used to distribute 2 drops of the cell suspension per well into 48 wells of a 96-well culture plate (Costar) and 1 drop per well into the other 48. One drop of complete medium plus feeder cells was added to each of the first 48 wells, and 2 drops were added to each of the other 48. Around 7 days after cloning the hybridomas could be seen by microscopy, and each well was scored for monoclonal hybridoma.

**Indirect ELISA.** Hybridomas secreting specific antibodies against Bp-ST were detected by indirect enzyme-linked immunosorbent assay (ELISA), as described by Banchun et al. (3) with a slight modification, using panel protein antigens of S. typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C, S. choleraesuis, S. enteritidis, S. kentf, S. panama, S. typhimurium, E. coli, P. pseudomallei, and Y. enterocolitica. Briefly, protein antigens were coated at 10 μg/ml onto Microelisa Immulon plates (Dynatech Laboratories, Inc., Alexandria, Va.) by 5 h of incubation at 37°C. The plates were washed three times with 0.05% (vol/vol) Tween 20-saline and tapped dry. The culture supernatants were added, and the plates were incubated at 4°C overnight. After three washes with Tween-saline, alkaline phosphatase-conjugated goat anti-mouse polyvalent immunoglobulin (Southern Biotechnology, Birmingham, Ala.) was added to each well. The plates were incubated at 37°C for 3 h. After excess conjugate had been washed out, freshly prepared p-nitrophenylphosphate (Sigma Chemical Co., St. Louis, Mo.) was added at 1 mg/ml to each well. The reaction was allowed to take place at room temperature for 60 min and then was terminated by addition of 3 M NaOH. The optical density was measured immediately at 405 nm with a Titertek Multiskan (Flow Laboratory GmbH, Bonn, Federal Republic of Germany).

**Determination of MAb isotypes.** Culture supernatants of hybridomas secreting specific antibodies were characterized for their isotypes by indirect ELISA. The procedure was the same as described above, except that alkaline phosphatase-conjugated goat anti-mouse immunoglobulin M (IgM), IgG1, IgG2a, IgG2b, and IgG3, κ and λ chains (Southern Biotechnology) were used instead of the anti-mouse polyvalent immunoglobulin.

**Immunoblot techniques.** The protein antigens of 12 bacteria were analyzed with a 2001 vertical electrophoresis unit (LKB-Produktur AB, Bromma, Sweden) by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method originally described by Laemmli (10). After electrophoresis, the proteins were transferred to nitrocellulose by using a Transblot apparatus with a 15°C cooling system (LKB-Produktur AB) at 90 V overnight. The nitrocellulose membranes were washed with 1% (vol/vol) Tween 20-PBS (PBST) (pH 7.2) at 37°C for 30 min, and unoccupied binding sites were blocked by incubation in 3% (wt/vol) bovine serum albumin in PBST at room temperature for 60 min on a rotating platform. MAb's diluted in PBST were added to the membranes, which were then incubated at room temperature for 2 h. After the membranes were washed with PBST, alkaline phosphatase-conjugated goat anti-mouse polyvalent immunoglobulin (Dakopatts, Glostrup, Denmark) was added, and incubation took place for 60 min at room temperature. A red-purple pigment was obtained by incubating washed membranes in a mixture of 4 mg of β-naphthyl phosphate (Sigma) and 60 mg of o-dianisidine, tetrazotized (Sigma) (12, 15), for 5 min: the filter was rinsed with tap water to stop the reaction. The red-purple band on the nitrocellulose membrane was considered the positive or specific band; the negative or nonspecific-staining band appeared brownish yellow.

**Molecular weight determination.** The molecular weights of protein bands of S. typhi specific to both MAbs on the nitrocellulose filter membrane were determined by comparing the electrophoretic mobility of these bands with that of known protein markers as described by Weber and Osborn (16).

**Antibody production in vivo.** To obtain high yields of antibodies, we grew the hybridomas as ascites tumors in BALB/cj mice primed with pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Inc., Milwaukee, Wis.). Pristane (0.5 μl/mouse) was injected intraperitoneally into BALB/cj mice at 6 to 12 months of age. At 3 days to 2 weeks after the pristane injection, the mice were each given 2 × 10⁷ hybrid cells in 3 ml of PBS intraperitoneally. Ascitic fluid developed within 2 weeks, and 10 to 15 ml of fluid could be withdrawn from the peritoneal cavities of the mice several times.

**Modified double-antibody sandwich ELISA.** Both MAbs in the ascitic fluid were precipitated by the addition of saturated (NH₄)₂SO₄ to a final concentration of 50%. The precipitate was dialyzed against PBS (pH 7.2) for 48 h with five changes of buffer and then stored at −20°C until used in ELISA.

The purified MAbs (STP14 and STP13) were diluted in 0.05 M carbonate buffer (pH 9.8) containing 0.1% sodium azide to protein concentrations of 25 and 5 μg/ml, respectively. A 100-μl portion of the solution was added to each well of Microelisa Immulon plates, which were then incubated at 4°C overnight. After that, the plates were washed three times with PBST and tapped dry. A 100-μl portion of Bp-ST at various concentrations in PBST was added to each well, and the plates were incubated at 37°C for 3 h. Each specimen was assayed in duplicate. After three washes with PBST, 100 μl of the second antibody, which was rabbit anti-Veronal buffer extract of S. typhi (anti-VBE) immunoglobulin diluted in PBST to either 5 or 2.5 μg/ml, was added to each well. The first concentration was used with STP14, and the second concentration was used with STP13. The plates were then incubated at 30°C for 2 h. After they had been washed, 100 μl of goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate (Sigma) diluted 1:2,000 in PBST containing 1% bovine serum albumin was added to each well, and the plates were incubated at 4°C overnight. The excess conjugate was washed out three times with PBST, and 100 μl of substrate solution (p-nitrophenyl phosphate) was added at 1 mg/ml to each well. The reaction was allowed to take place at 37°C for 1 h and was stopped by the addition of 25 μl of 3 M NaOH. The ΔA₄₅₀ was read spectrophotometrically (Titertek Multiskan). In each assay, a negative control, conjugate control, and substrate blank were also included. PBST (100 μl) was substituted for the antigen in the negative control, for both the antigen and rabbit anti-VBE immunoglobulin in the conjugate control, and also for conjugate in the substrate blank. A total of eight assays were performed with each MAb, and Student’s t test was used for statistical analyses.

**RESULTS**

Indirect ELISA with a panel of 12 Bp antigens was used to select two hybridomas producing specific antibodies only to
the protein antigen of *S. typhi* for further investigation. Antibody STP13, which originated from the lymph node fusion, showed an IgGlκ isotype. Antibody STP14, which was derived from the spleen fusion, had an IgG1κ isotype.

**Demonstration of specific protein bands by immunoblotting.**

The separated protein bands of *S. typhi*, *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. choleraesuis*, *S. enteritidis*, *S. kelfeld*, *S. panama*, *S. typhimurium*, *E. coli*, *P. pseudomallei*, and *Y. enterocolitica* on nitrocellulose filter membranes were stained with immunoenzyme by using MAbs STP13 and STP14 as the specific antibodies. Each MAb reacted only with one protein band of *S. typhi* (Fig. 1). The molecular masses of the reacted bands were determined from a standard curve of Coomassie blue-stained protein markers on the same filter membrane, which revealed that both bands had the same molecular mass (34.0 kDa) and the same relative mobility (0.51). The *S. typhi* protein band which reacted with MAb STP14 was wider than the band which reacted with MAb STP13.

MAbs STP13 and STP14 could react very weakly with a band of protein of approximately the same molecular mass from *S. paratyphi* C, *S. choleraesuis*, and *S. typhimurium* (Fig. 2 to 4), although the weak reactions could not be seen in indirect ELISA.

The presence of this 34.0-kDa protein band was also demonstrated when the separated protein bands of *S. typhi* were reacted with serum samples from the patients with typhoid fever and from the vaccinees who had received parenteral acetone-inactivated or heat-inactivated phenol-preserved typhoid vaccines or parenteral live attenuated *S. typhi* Ty2la vaccine.

**Antigen detection by modified double-antibody sandwich ELISA.**

MAbs STP13 and STP14 were experimentally designed for detection of Bp-ST at various concentrations. The results show that both STP13 and STP14 could detect the protein antigen of *S. typhi* at a concentration of 0.6 μg/ml (*P* < 0.005) (Fig. 5).

**DISCUSSION**

Two MAbs, STP13 and STP14, were established. By the immunoblotting technique, each clone was shown to be specific to a 34.0-kDa protein band of *S. typhi*. The patterns of the staining bands were not exactly the same: the band that reacted with STP14 was wider than the band that reacted with STP13, which suggests that both MAbs are not exactly specific to the same epitope of the protein antigen, since the band separated by SDS-PAGE may contain proteins with the same molecular weight but different amino acid compositions.

To confirm the specificity of these protein-specific MAbs, immunoblotting was performed with a panel of antigens from 12 bacteria. The results showed unexpectedly weak reactions of both MAbs with Bp of *S. paratyphi* C, *S. choleraesuis*, and *S. typhimurium*. These cross-reactivities were not found in an indirect ELISA. Possible explanations are (i) that hidden epitopes of Bp-ST are exposed after protein has been denatured.
been subjected to denaturing conditions during SDS-PAGE or (ii) that nitrocellulose has a much greater capacity for protein binding per unit area than does polystyrene, leading to a significant increase in the sensitivity of immunoblotting when compared with the indirect solid-phase ELISA.

The detection of the 34.0-kDa protein antigen of *S. typhi* by STP13 and STP14 MAbs should be very useful for serodiagnosis of patients with typhoid fever. The weak cross-reactivities with protein antigens of *S. paratyphi* C, *S. choleraesuis*, and *S. typhimurium* in the immunoblot are so minimal that they should not interfere with other, less sensitive, conventional tests used in routine diagnosis and should not be considered a great disadvantage of these MAbs. With regard to the effect of the test on treatment of patients, these cross-reactivities should not interfere in the plan of treatment, because antibiotic therapies with these four bacteria are very similar (5).

In determining the ability of the established MAbs for *S. typhi* antigen detection, modified double-antibody sandwich ELISA was performed with various concentrations of Bp-ST. STP14 and STP13 could detect very small amounts of Bp-ST at ca. 0.6 µg/ml. Their sensitivities are comparable to that of the polyclonal rabbit anti-Bp-ST in our previous report, which showed that the minimal detectable level was 0.5 µg of Bp-ST per ml (2). However, in the work described in that report, cross-reactivities of polyclonal rabbit anti-Bp-ST with other, related enteric bacteria were observed. In view of these results, the greater specificity of both MAbs gives them considerable potential as reagents in *S. typhi* antigen detection.

The evidence that the 54.0-kDa protein band was demonstrated when Bp-ST was reacted with serum samples from typhoid patients and vaccinees who had received different kinds of typhoid vaccine suggests that the 34.0-kDa protein antigen plays a role in the immune response against *S. typhi*. Hence, purified *S. typhi* 34.0-kd protein is an essential structural probe for dissecting the basic mechanisms of the immune response to this organism and also should have potential application as a specific skin test reagent for testing the cell-mediated immune response to *S. typhi* in vivo. Further studies concerning the purification and characterization of this 34.0-kDa protein by using MAbs STP13 and STP14, as well as clinical trials of both MAbs with the specimens from the patients, are in progress in our laboratory.

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LITERATURE CITED


