Production of Specific Monoclonal Antibodies to *Salmonella typhi* Flagellin and Possible Application to Immunodiagnosis of Typhoid Fever

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Four murine monoclonal antibodies (MAbs) to *Salmonella typhi* flagellin were produced. These MAbs did not react with eight other enterobacterial strains tested: *Salmonella enteritidis, Salmonella typhimurium, Salmonella paratyphi A, Escherichia coli, Shigella flexneri, Shigella sonnei, Yersinia enterocolitica*, and *Campylobacter jejuni*. All four MAbs cross-reacted with *Salmonella muenchen* flagellin indicating specificity for d antigenic flagellar epitope. One MAb (C4) was selected to develop a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) to detect *S. typhi* flagellin in serum samples. By use of this assay *S. typhi* flagellar antigen was detected in 95.5% of serum samples from patients with positive hemoculture for *S. typhi*, in 93.6% of samples from patients with positive serodiagnosis of typhoid fever, in 26% of samples collected from patients who were initially hemoculture-positive for *S. typhi* and who had undergone 7-8 d of chemotherapy, in 8.5% of samples from healthy persons from an endemic area, and in no samples from healthy persons from a nonendemic area. The presence of high levels of flagellin antibody titers did not interfere with the antigen detection. The detection of *S. typhi* flagellar antigen in patient serum may have practical value for rapid diagnosis of typhoid fever.

In many parts of the world typhoid fever remains an important public health problem [1]. *Salmonella typhi* infection may be asymptomatic or cause overt disease in young children or adults. Early, rapid, specific, and sensitive diagnosis of typhoid fever is important for prompt and effective therapy. The conventional methods of diagnosis are bacterial culture and antibody detection. Hemocultures are positive for *S. typhi* in about 80% of patients during the first week of illness. Bone marrow cultures are positive in about 90% or more patients from the first week. Coproculture becomes positive only later in up to 80% of patients, but remains positive for long periods. Urine culture is least often positive; however, those cultures take 2-5 days and require microbiologic laboratory facilities, which are not always available in areas where typhoid fever is endemic. Antibody measurement of a single sample is not helpful in endemic areas [2, 3], and the detection of rising antibody titers is too slow to allow a quick decision by the clinician. Therefore, alternative methods for a rapid diagnosis of typhoid fever are needed. Efforts were made to develop such methods using mainly polyclonal antibodies to *Salmonella* to detect bacterial antigens in blood [4-9], urine [10-13], or feces [14, 15]. To date the main limitation has been the lack of specificity of the polyclonal antibodies used. Recently, monoclonal antibodies (MAbs) have been used in an attempt to develop antigen detection tests [16-24].

After developing MAbs specific for *S. typhi* flagellin, we assessed their potential value as diagnostic tools for the immunologic detection of *S. typhi* antigen in serum from patients with typhoid fever.

Materials and Methods

All enterobacterial strains used in this study were donated by Prof. L. Le Minor, World Health Organization Reference Centre, Bern, Switzerland, or obtained from the University Cantonal Hospital, Geneva. The following species were used: *S. typhi* (E. 83. 714, E. 83. 724, E. 83. 728, E. 83. 733, E. 83. 738.), *Salmonella enteritidis, Salmonella paratyphi A, Salmonella muenchen, Salmonella typhimurium, Shigella flexneri, Shigella sonnei, Yersinia enterocolitica*, and *Campylobacter jejuni*. All *S. typhi* strains were Vi positive. The E. 83. 728 *S. typhi* strain culture was used for the purification of flagellin.

Cultures. The stab cultures were maintained in nutrient agar. The maximum motility was obtained by serial passage through semisolid medium: casitone 10.0, yeast extract 3.0, sodium hydroxide 5.0, bacto-agar 3.0 (g/l of distilled water). The bottom part of the last semisolid medium passage was inoculated in a broth culture composed of equal parts of trypticase soy broth (BBL Microbiology Systems, Becton Dickinson, Cockeysville, MD) and tryptose broth (Difco Laboratories, Detroit), then incubated at 37°C for 12-15 h. An equal volume of saline containing 0.5% formalin was added, and the broth culture was refrigerated and used as a test antigen.

Isolation of S. typhi flagellin. *S. typhi* flagellin was purified according to the method of Ibrahim et al. [25]. Flagella were detached by exposure of bacteria to 1 N hydrochloric acid (pH 2), then centrifuged at 100,000 g for 1 h at 4°C. The supernatant containing flagellin was adjusted to pH 7.2 with 1 N sodium hydroxide, precipitated

...concentration of 2.67 M, and left for ... precipitated flagellin was separated by centrifugation ... for 15 min and dialyzed against water.

Purity of flagellin was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was carried out in 0.1% SDS polyacrylamide slab gels by using the Tris-glycine discontinuous buffer system of Laemmli [26] supplemented with 0.5 M urea. Stacking and resolution gels were at 5% and 10% acrylamide, respectively. Each gel lane was loaded with 5–15 µg of flagellin and was run at 40 mA constant current per gel slab at room temperature for 3 h. Proteins were stained with Coomassie brilliant blue, and molecular weights were established with molecular weight standards of 14–200 kDa (BRL Laboratories, Bethesda, MD).

Production of rabbit antibody to S. typhi flagellin. Antibodies to flagellin were raised in a female New Zealand white rabbit (bred at our animal facility) by injection of 100 µg of purified flagellin in complete Freund's adjuvant (CFA) followed by three successive injections of 50 µg of flagellin in incomplete Freund's adjuvant (IFA) 20, 27, and 31 days later. Serum titers of antibodies to *S. typhi* flagellin were determined by tube agglutination (Widal test). Rabbit preimmune serum served as the control. Rabbit antibodies to *S. typhi* flagellin IgG were purified by anion exchange chromatography (DE52, Pre-Swollen Microgranular Anion Exchanger; Whatman, Maidstone, Kent, UK). Fractions collected were tested in immunoelectrophoresis against a sheep antibody to rabbit IgG (Cappel Laboratories, Cochranville, PA).

Production and characterization of S. typhi flagellin MAbs. For the production of MAbs, female 6–8-week-old BALB/c mice were used. The original pairs of these mice originated at the Jackson Laboratory, Bar Harbor, ME. Six mice were immunized intraperitoneally (ip) and subcutaneously (sc) with 100 µg of *S. typhi* flagellin in CFA. Twelve days later they were boosted ip with 50 µg of flagellin in IFA. A third ip immunization with 50 µg of flagellin in saline was done 31 days later. *S. typhi* flagellin antibody response was checked by ELISA (see below). Three days after the last injection, the spleen was removed and fused with the mouse P3-X63/Ag 8 myeloma cell line [27]. Hybridomas producing *S. typhi* flagellin antibodies were screened by ELISA with purified *S. typhi* flagellin antigen. Positive cultures were cloned three times by limiting dilution (0.3 cells/well). Clones A1, C4, F8, and H10 producing the highest titers of antibodies were selected, expanded, and then injected ip into pristane-treated BALB/c mice. MAbs were partially purified from ascites by precipitation with 50% ammonium sulfate. MAb isotypes were determined by ELISA using rabbit antibodies to mouse IgG1, IgG2a, IgG2b, and IgG3 (Litton Bionetics, Kensington, MD) and goat antisera to mouse IgM (Cappel) conjugated to alkaline phosphatase.

Immunoblotting. Purified flagellin was subjected to SDS-PAGE in 12% acrylamide gels containing 0.1% SDS and 0.5 M urea. Proteins were electroblotted onto nitrocellulose paper in methanol-Tris-glycine buffer at 6 V/cm for 16 h as described by Towbin et al. [28]. After probing with ascites diluted 1:100–1:500, the strips were washed, incubated with 1:1000 dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO a/s, Copenhagen) and washed again. Specific bands were developed with 4-chloro-1-naphtol (Merck Laboratories, Zurich; 0.6 mg/ml) in Tris-buffered saline-methanol buffer containing 0.03% H_2O_2.

Indirect ELISA for detection of antibodies to S. typhi flagel-
lin. Flat-bottom 96-well plates (Immunoplate 1; Nunc, Roskilde, Denmark) were coated with 10 µg/ml of purified *S. typhi* flagellin or different strains of formalin *S. typhi* (10^7–10^8 bacteria/ml) diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 37°C for 3 h. Wells were saturated for 2 h with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 (PBS-T). Mouse sera, culture supernatants, and ascites were tested at different dilutions by incubation at room temperature for 2 h. After washing with PBS-T, binding of antibodies was detected by an alkaline phosphatase–conjugated goat antibody to mouse IgG, 1 µg/ml in PBS-T with 1% BSA at room temperature for 2 h. After further washes with PBS-T, the enzymatic reaction was revealed by adding, as a substrate, *p*-nitrophenylphosphate (Sigma Chemical, St. Louis) 1 mg/ml in 0.01 M diethanolamine solution. The optical density was read at A_{405} with a Titertek Multiskan reader (Flow Laboratories, McLean, VA).

The detection of human antibodies to *S. typhi* flagellin in the patients' serum was carried out as described above, with samples diluted 1:200, 1:400, 1:800, 1:1600, and 1:3200. Alkaline phosphatase–conjugated goat antibody to human IgG (TAGO, Burlingame, CA) was used at a 1:100 dilution.

Competitive ELISA to define antigen specificity of the S. typhi MAbs. In some experiments, fixed concentrations of one antibody to *S. typhi* flagellin MAb C4 (5 µg/ml), conjugated to alkaline phosphatase according to the method described by Avrameas [29], were mixed with different dilutions of the same or other unconjugated anti-flagellin MAbs. Then 50 µl of these mixtures was tested by ELISA as described above.

Clinical samples. Serum specimens were collected at El Kettar Hospital, Algiers, Algeria, from four groups of patients and kept at −20°C until use. Samples included 69 collected from patients immediately after admission and tested for signs of typhoid fever. These patients were subsequently screened for microbiologic or clinical diagnoses: 22 had positive hemoculture for *S. typhi* (group 1), and 47 had negative hemoculture but were positive for antibodies to *S. typhi* flagella with titers of 400–3200 (group 2). Fifteen serum samples from patients with culture positive for typhoid fever were collected 1 week after the beginning of chemotherapy (group 3), and 35 serum samples were from healthy individuals (group 4). A fifth group was 15 serum samples from normal, healthy individuals collected at Cantonal Hospital, Geneva. Details are summarized in table 1.

Sandwich ELISA assays for the detection of S. typhi flagellin in human serum. Flat-bottom 96-well plates (Nunc) were coated by overnight incubation at 37°C with 50 µl of rabbit IgG antibody to *S. typhi* flagellin at 50 µg/ml in carbonate buffer, 0.05 M (pH 9.8). After three washes with PBS-T, plates were saturated for 2 h at room temperature with 50 µl of PBS-T containing 1% BSA (Sigma). Then 50 µl of serum diluted 1:5 in PBS-T with 1% BSA was added to duplicate wells and incubated for 2 h at room temperature. After three washes with PBS-T, the presence of antigen was detected by adding alkaline phosphatase conjugated *S. typhi* flagellin MAb C4 (5 µg/ml) in PBS-T with 1% BSA. The plates were incubated for 2 h at room temperature. After three washes with PBS-T, the substrate solution *p*-nitrophenylphosphate (Sigma) was added. The results were read at A_{405} and expressed in terms of flagellar antigen concentration with reference to a standard curve obtained with purified *S. typhi* flagellin.

Table 1. Findings of clinical samples tested for *Salmonella typhi* flagellin antigen by sandwich ELISA.

Group	No. of patients	Range of S. typhi flagellin antibody titers*	No. of patients with detectable levels of S. typhi flagellin (%)[†]
Typhoid fever			
HC pos; AB no	22	800–3200	21 (95.4)
HC neg or no; AB no	47	400–3200	44 (93.6)
HC pos; AB yes	15	400–3200	4 (26.6)
Healthy controls			
Endemic area	35	<200	3 (8.5)
Nonendemic area	15	<200	0 (0)

NOTE. HC, hemoculture: positive (pos), negative (neg), or not done (no). AB, antibiotic treatment: (yes) ampicillin and/or chloramphenicol for 1 week before sample collection, (no) without treatment.

* S. typhi flagellin antibody titers as measured by ELISA using purified S. typhi flagellin as solid phase.

[†] Serum samples were considered positive when >5 ng/ml of S. typhi flagellin antigen was detected.

Results

Analysis of purified S. typhi flagellar protein. Purified *S. typhi* flagellin analyzed by SDS-PAGE revealed one major Coomassie brilliant blue band of 52 kDa. This antigen preparation was used to immunize mice and rabbits (figure 1, lane 2).

Production of rabbit polyclonal and murine MAbs to S. typhi flagellar antigen. Rabbit immunized with *S. typhi* flagellin produced high levels of H antibodies, which cross-reacted with *S. enteritidis* flagellin; this cross-reaction was also observed in mouse serum immunized with purified *S. typhi* flagellin (not shown). The mouse producing the highest titer of antibodies to *S. typhi* flagellin was killed for the production of monoclonal antibodies. After cloning three times, four stable clones secreting high levels of *S. typhi* flagellin IgG1 MAbs were obtained.

Specificity of MAbs against S. typhi flagellin. An indirect ELISA test was used to assess the reactivity of MAbs to a variety of enterobacterial species. The four MAbs reacted with flagellin purified from two different strains of *S. typhi*, with all 6 *S. typhi* isolates, and with 2 *S. muenchen* isolates. These MAbs did not react with 6 isolates from other *Salmonella* species or with 10 isolates of enterobacteria (table 2). These results indicate an exquisite specificity of these MAbs for d flagellin antigen. Of the four MAbs tested, C4 showed the highest binding to *S. typhi* flagellin.

By immunoblotting, these MAbs were shown to react with the major 52-kDa protein of the flagellin preparation (figure 1, lanes 3–5). To ascertain whether the four MAbs were directed against the same or different epitopes of *S. typhi* flagellin, a competitive ELISA was carried out. We have shown that the binding of alkaline phosphatase–conjugated MAb C4 to *S. typhi* flagellin was completely inhibited by an excess of
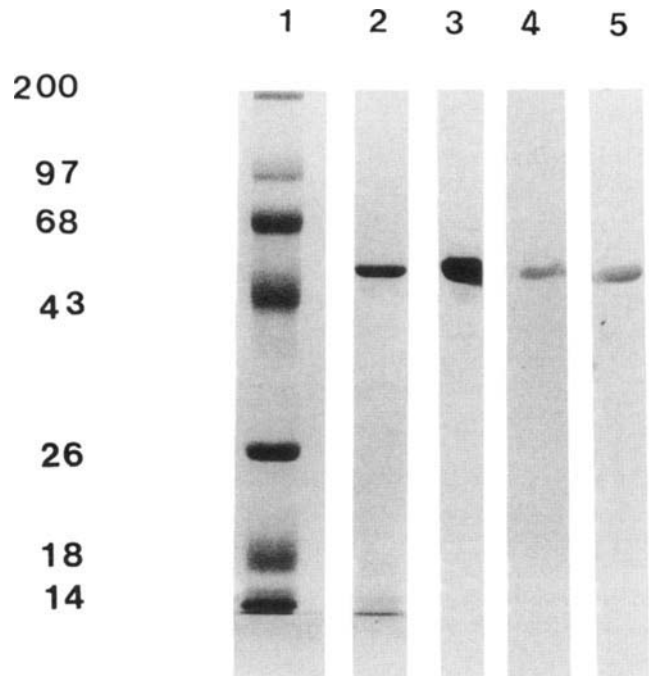

Figure 1. SDS-PAGE and immunoblotting analysis of purified *Salmonella typhi* flagellin. Purified *S. typhi* flagellin was electrophoresed in SDS-polyacrylamide gels (5 µg/lane) and stained with Coomassie brilliant blue (lane 2) or probed with MAbs A1 (lane 3), C4 (lane 4), and F8 (lane 5). Binding of MAbs to the antigen was revealed by incubation with a peroxidase-conjugated rabbit IgG anti-mouse immunoglobulin and then with 4-chloro-1-naphtol as a substrate. Molecular weight standards stained with Coomassie blue, lane 1.

unconjugated MAb C4 and by the MAb H10. MAb A1 inhibited the binding of MAb C4 to a lesser extent, but MAb F8 did not block the binding of C4 to *S. typhi* flagellin. This suggests that MAbs C4 and H10 recognize the same epitope, which differs from that recognized by MAb F8.

Application of S. typhi MAbs for diagnosis of typhoid fever. An ELISA was developed to detect soluble *S. typhi* flagellin. Rabbit *S. typhi* IgG antibodies were used in solid phase to capture the flagellin antigen and the alkaline phosphatase–conjugated MAb C4 was used as the probe antibody.

In preliminary experiments using purified *S. typhi* flagellin at different dilutions in normal human serum, it was found that this assay could detect 5–10 ng/ml of *S. typhi* flagellin diluted in normal human serum. As expected, *S. enteritidis* flagellin gave negative results (figure 2). The coefficient of variation is ∿6%, suggesting a good reproducibility of this test.

To evaluate the specificity of the assay, the same sandwich ELISA was performed as described above using intact bacteria in suspension. Only *S. typhi* and *S. muenchen* were recognized by MAb C4; no binding was revealed with *S. paratyphi A*, *S. typhimurium*, *S. enteritidis*, *Sh. flexneri*, *Sh. son-*

Table 2. Reactivity of *Salmonella typhi* flagellin monoclonal antibodies to *S. typhi* flagellin, *S. typhi* bacteria, and other related organisms by indirect ELISA.

Antigens,* group	H. antigen specificity	No. of strains tested	No. of tested strains reacting with monoclonal antibody			
			A1	C4	F8	H10
S. typhi flagella		2	2	2	2	2
S. typhi, D	d:—	6	6	6	6	6
S. muenchen, C	d:1,2	2	2	2	2	2
S. enteritidis, D	g,m:1,7	2	0	0	0	0
S. paratyphi A, A	a:—	2	0	0	0	0
S. typhimurium, B	i:1,2	2	0	0	0	0
Sh. flexneri		2	0	0	0	0
Sh. sonnei		2	0	0	0	0
Y. enterocolitica		2	0	0	0	0
C. jejuni		2	0	0	0	0
E. coli		2	0	0	0	0

* Purified *S. typhi* flagellin was used at 10 μg/ml, formalin killed bacteria were used at 10^8 bacteria-ml, as solid phase. All organisms were isolated from patients with diarrheal disease.

nei, *Y. enterocolitica*, *C. jejuni*, or *Escherichia coli* (not shown).

The sandwich ELISA was applied to the detection of *S. typhi* flagellar antigen in serum samples from patients with typhoid fever. Of the 69 samples collected before therapy (samples from patients with microbiologically or serologically diagnosed typhoid fever), 94.5% had detectable levels of *S. typhi* flagellin in the serum. Of the 22 samples, 21 (95.4%) from patients with positive hemoculture had *S. typhi* flagellin antigen in serum (table 1). All serum samples were positive for *S. typhi* flagellin antibodies by ELISA with titers ranging from 1:400–1:3200. Four (26%) of 15 samples from typhoid fever patients collected 1 week after starting chemotherapy still had detectable amounts of *S. typhi* flagellin (figure 3). Three of the 35 samples (8.5%) from healthy individuals from an endemic area gave results (range, 5.2–5.4 ng/ml) just above the limit of detection. All of the control serum samples from healthy blood donors from nonendemic areas gave negative results. The results of the antigen detection test were plotted against the *S. typhi* flagellin antibody levels (not shown). It appears that presence of *S. typhi* flagellin antibodies does not interfere with the detection of serum *S. typhi* flagellin using MAb C4.

Discussion

In the present study highly specific monoclonal antibodies to *S. typhi* flagellin were produced, and we assessed their possible use as immunodiagnostic reagents for the diagnosis of typhoid fever. These MAbs were selected on the basis of a high specificity restricted to epitopes on the 52-kDa *S. typhi* flagellin antigen characteristic of the d flagellar specificity.
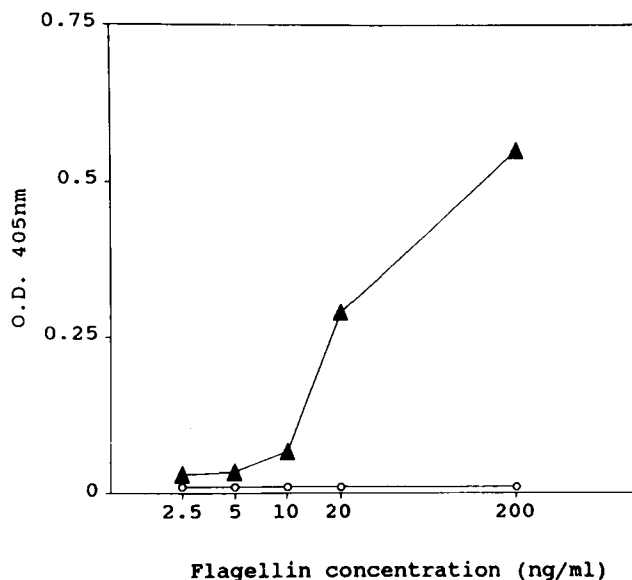

Figure 2. Assessment of the sensitivity of sandwich ELISA for the detection of *Salmonella typhi* flagellin antigen. Microwells were coated with rabbit anti-*S. typhi* flagellin (50 μg/ml) and then reacted with normal human serum containing different concentrations of *S. typhi* flagellin (▲) or *S. enteritidis* flagellin (O). The presence of antigen was detected with the C4 MAb anti-*S. typhi* flagellin (5 μg/ml) conjugated to alkaline phosphatase. Results are expressed as the mean optical density at 405 nm in duplicate wells.
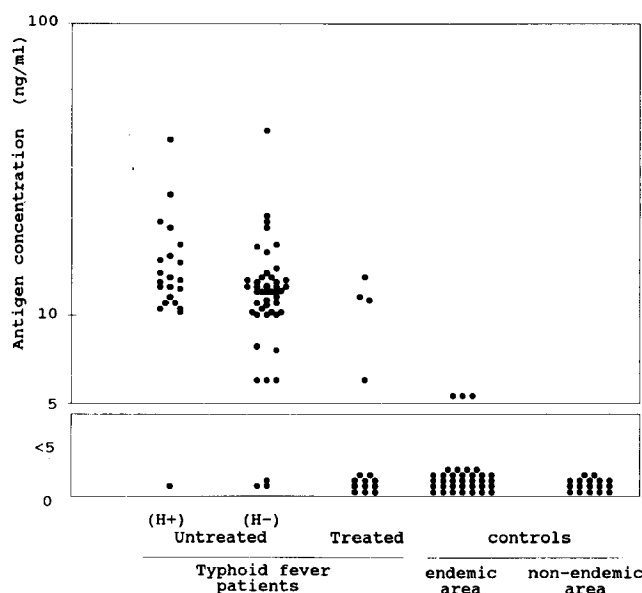

Figure 3. Detection of *Salmonella typhi* flagellin antigen in serum from four groups of subjects: untreated typhoid fever patients with positive hemoculture (H+, *n* = 22) or negative hemoculture (H−, *n* = 47); typhoid fever patients treated 1 week (*n* = 15); and normal controls from endemic (*n* = 35) and nonendemic (*n* = 15) areas. Each point represents the mean of duplicate wells. Results are expressed in terms of flagellar antigen concentration with reference to a standard curve obtained with purified *S. typhi* flagellin.

The d flagellar antigen is characteristic of *S. typhi* and a few rare *Salmonella* species of which *S. muenchen* is the most prevalent. Our studies confirmed the reactivity of MAbs C4, A1, H10, and F8 with *S. muenchen* or *S. muenchen* flagellin, as expected from structural data on this antigen [30]; however, this cross-reactivity is of relatively little importance since *S. muenchen* infections are infrequent and usually are not a problem for the differential diagnosis of typhoid fever.

The sandwich test was developed to detect flagellin in patient serum using C4, the most potent MAb, and rabbit antibody to flagellin as capture antibody. This test appears to be quite sensitive with a limit of detection of about 5–10 ng/ml. The sensitivity of this test was not higher when each MAb was used as a solid-phase capture antibody (not shown). The limit of detection for whole formalin–treated bacteria was 10^4–10^5 bacteria/ml.

The results obtained when this test was applied to serum samples from Algerian patients with typhoid fever indicate the possible usefulness of an antigen detection test using highly specific MAbs (table 1). Of 22 patients with positive hemoculture, 95.4% were positive by sandwich ELISA. The presence in patient serum of antibodies to *S. typhi* flagellin (with titers of 400–3200) did not interfere with the detection of the *S. typhi* flagellin antigen. Possibly the presence of immune complexes in serum does not affect the accessibility of the epitope on flagellin recognized by MAb C4. Human antibodies produced against *S. typhi* flagellin after natural infections may recognize epitopes different from that recognized by MAb C4. Our preliminary results showed that 3 (8.5%) of 35 serum samples from apparently healthy individuals from an endemic area gave results just above the limit of detection; control serum from nonendemic areas was always negative. Many more samples from normal individuals or from patients with other enteric infections or with other causes of fever must be tested. Bacteremia occurring in patients with typhoid fever is low, ~10–20 bacteria/ml. This value is 1000 times lower than the limit of detection of our ELISA test using whole bacteria, suggesting that this assay detects antigenemia rather than bacteremia during the acute phase of typhoid fever.

There have been previous attempts to develop tests to detect *S. typhi* antigen(s) in human specimens. These tests, counterimmunoelectrophoresis [7, 9, 13], coagglutination [6, 7, 12, 13, 15], and ELISA [4, 5, 10], were largely based on the use of polyclonal antibodies to *Salmonella* to detect bacteria or antigens in blood, urine, or feces. The sensitivity and specificity were not sufficient for their use as diagnostic tests. The nature of the probe antibody used and time of sample collection probably explained the variations in the sensitivity and specificity reported. More recently, there have been reports of monoclonal antibodies directed against different *Salmonella* antigens, such as O9 [16, 17], Vi [23, 24], and barber protein Bp [21], and of antibodies that bind to flagellar determinants of the organism but that are not directed toward the H antigen [19, 20, 22]. The anti-Vi MAbs [24] cross-react only with *S.*

dublin and *Citrobacter*, the anti-O9 MAbs [16, 17] cross-react with all group D *Salmonellae*, *S. panama*, and *S. enteritidis*, and the anti-Bp MAbs [21] cross-react with *S. paratyphi C*, *S. choleraesuis*, and *S. typhimurium*. Finally, the two antibodies that bind to flagellar determinants [19, 20, 22] were reported to detect all *Salmonella* organisms in food and stool samples.

In view of the antibodies reported in the literature, the potential use of highly specific MAbs for the immunodetection of flagellar antigen in human samples should be seriously considered as a tool for the early diagnosis of typhoid fever. Although such assays do not replace diagnosis by bacterial culture, they provide results on the day of admission and may be useful in developing countries where facilities for bacterial cultures are often absent.

The production of similar highly specific MAbs against flagellar epitopes from other important *Salmonella* species, such as *S. paratyphi A*, is now in progress in our laboratory. We hope that a combination of these reagents may be particularly useful in the immunodiagnosis of typhoid and paratyphoid fever.

Acknowledgment

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