Journal of Clinical Microbiology, Sept. 1988, p. 1824–1830
0095-1137/88/091824-07$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 26, No. 9

Detection with Monoclonal Antibody of *Salmonella typhi* Antigen 9 in Specimens from Patients

WANPEN CHAICUMPA,[1]* WUTTIPONG THIN-INTA,[1] SRISIN KHUSMITH,[1] PRAMUAN TAPCHAISRI,[1] PETER ECHEVERRIA,[2] THAREERAT KALAMBAHETI,[1] AND MANAS CHONGSA-NGUAN[1]

Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road,[1] and Armed Forces Research Institute of Medical Science, U.S. Component,[2] Bangkok 10400, Thailand

Received 1 February 1988/Accepted 26 May 1988

Monoclonal antibodies were raised against Barber antigen (Ba) of *Salmonella typhi* 0901. Antibodies produced to antigen 9 of group D salmonellae were used in double- and triple-sandwich antibody enzyme-linked immunosorbent assays (ELISAs) for detecting antigen 9 in urine and plasma specimens from three groups of patients and 49 controls. The triple-antibody ELISA detected the antigen in urine samples from 11 of 18 (65%) patients with hemoculture-proven typhoid (group 1) and 12 of 39 (31%) patients with clinical features compatible with typhoid but whose hemocultures were negative (group 2). This ELISA was negative in three patients from whom *Salmonella paratyphi* A, *Escherichia coli*, and *Klebsiella pneumoniae* (group 3) were isolated by hemoculture and in all healthy controls. The double-antibody sandwich ELISA was positive in 41 and 15% of urine samples from patients in groups 1 and 2, respectively, and was negative with samples from two patients from group 3 and all controls. The sensitivity and specificity compared with those for healthy controls were 65 and 100%, respectively, for the triple-antibody ELISA. Although as little as 7.8 ng of homologous lipopolysaccharide could be detected, background in clinical specimens prevented accurate interpretation of the detection of this antigen in serum. Results were best with urine specimens.

Typhoid fever is still a public health problem in developing countries. There are an estimated 7 million cases of typhoid fever per year in south and east Asia where there are about 1,369 million people (5). The incidence of typhoid fever is approximately 540 cases per 100,000 people. Recent attention has been focused on new immunological methods for the diagnosis of typhoid fever by detection of an antigen(s) of *Salmonella typhi* in biological fluids of patients. Several serological tests have been developed, including passive staphylococcal coagglutination (9, 15–17) and counterimmunoelectrophoresis (7, 17, 18). However, counterimmunoelectrophoresis was found to lack specificity and has not been useful for diagnosing typhoid fever, while the reagents for the coagglutination test need careful standardization. Enzyme-linked immunosorbent assays (ELISAs) with polyclonal antibodies have also been developed for detecting Vi and proteins of *S. typhi* in body fluids of patients (1, 20). However, these ELISAs were not reliable since positive results were found with other systemic infections, particularly paratyphoid fever and systemic infections caused by other members of the family *Enterobacteriaceae*. The low specificity was thought to originate from the lack of specificity of the immunoreactants used in the systems. The polyclonal antisera produced against the desired antigens of *S. typhi* (i.e., anti-Vi, anti-Barber antigens [Ba]) might well include antibodies to other antigens (i.e., O, H, proteins) common to other salmonellae as well as to other members of the *Enterobacteriaceae*. When these antisera were used in the tests, by either coating the wells of ELISA plates or conjugating them with enzyme, the antibodies in the sera would also bind to heterologous antigens causing a false-positive ELISA result. Monoclonal antibodies directed against specific antigens of *S. typhi* might improve the specificity of the systems.

Production of monoclonal antibodies specific to antigen 9 of *S. typhi* (group D) and the use of these antibodies in ELISAs are reported here.

MATERIALS AND METHODS

Bacterial strains. *S. typhi* 0901; other salmonellae, namely, *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. typhimurium*, *Salmonella* group C_2, and *Salmonella* group E_4; and other gram-negative bacilli including *Escherichia coli*, *Shigella sonnei*, *Shigella flexneri*, and *Vibrio cholerae* were from lyophilized stock cultures of the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Fourteen clinical isolates of *S. typhi* and three isolates of *Salmonella panama* were obtained from the Department of Bacteriology, Armed Forces Research Institute of Medical Science, U.S. Component, Bangkok, Thailand.

Subjects. Plasma, urine, and blood samples were simultaneously collected from Khmer refugee patients with clinical features compatible with typhoid fever. These patients were admitted to the Khmer Refugee Camp Hospital in Aranyaprathet, Thailand, which was about 160 km east of Bangkok. The specimens were later classified into three groups according to the results of hemocultures. These were bacteriologically confirmed typhoid cases (group 1), presumptive typhoid cases (group 2), and other gram-negative bacteremic cases (group 3). Control plasma was obtained from the Blood Bank Unit, Ramadhibodi Hospital, while control urine samples were from the staff members of the Faculty of Tropical Medicine, Bangkok. The specimen collection period was from May 1986 to April 1987. All plasma and urine specimens were kept frozen at −70°C until assayed in July 1987.

Preparation of antigens. Ba were prepared from acetone-dried bacteria as previously described (2). Whole-cell antigens (WC) were prepared by suspending the bacteria in distilled water to an optical density (OD) of 2.0 at 540 nm and subjecting them to an MSE ultrasonicator at a maximum input for 5 min twice. Lipopolysaccharides (LPS) were

* Corresponding author.

extracted from acetone-dried bacteria by the hot phenol-water method (24). The single-extracted LPS was reextracted two more times to yield double-extracted and triple-extracted LPS (TE-LPS), respectively. Quantitative and qualitative determinations of proteins in the antigen preparations were performed by the method of Lowry et al. (13) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (11).

Preparation of rabbit anti-Ba immunoglobulins. A rabbit weighing 3 kg was immunized intramuscularly with 1 mg (dry weight) of Ba in 0.5 ml of normal saline solution mixed with an equal volume of Freund complete adjuvant and immunized 2 weeks later with the same dose of the antigen in Freund incomplete adjuvant. Sera obtained 1 week after the booster immunization had a high antibody level as assessed by indirect ELISA with goat anti-rabbit immunoglobulin-peroxidase conjugate (Dakopatts, Copenhagen, Denmark). The total immunoglobulins were separated by ammonium sulfate precipitation.

Immunization of mice. BALB/c mice were immunized intraperitoneally with 0.1 mg of Ba in 0.5 ml of normal saline solution mixed with an equal volume of Freund complete adjuvant, and 2 weeks later they were immunized with the same dose of antigen mixed with an equal volume of Freund incomplete adjuvant. The mice were boosted intravenously with 0.05 mg of Ba in 0.2 ml of normal saline solution 3 days before fusion of spleen cells with myeloma cells (Sp 2/0). Just before the mice were killed to collect spleen cells, they were bled from the retro-orbital plexi and their sera were pooled and used as a positive control in the indirect ELISA.

SDS-PAGE. SDS–PAGE was performed as described by Laemmli (11).

Western blot analysis. Western blotting (immunoblotting) was performed as described by Burnette (3) with modifications. The antigens were fractionated by SDS-PAGE and electroblotted onto nitrocellulose paper. The nitrocellulose sheet was submerged in blocking solution (2% bovine serum albumin in phosphate-buffered saline [PBS]) for 30 min, washed in PBS containing 0.05% Tween 20 and 0.2% gelatin to remove excess bovine serum albumin, and then allowed to react with the antibodies for 1 h at 25°C on a rocking platform. After washing, the sheet was put in a solution containing 5×10^5 cpm of ^{125}I-protein A per ml. Staphylococcal protein A (Pharmacia, Uppsala, Sweden) was radiolabeled with ^{125}I by the iodogen method described by Paus et al. (14). The ^{125}I-protein A was bound to the antigen-antibody complexes at 25°C for 30 min. The nitrocellulose paper was washed to remove unbound ^{125}I-protein A and dried on filter paper. After the paper dried, autoradiography was performed by placing the nitrocellulose on a paper screen, wrapping it in Glad-Wrap, and exposing it at −70°C to Kodak X-Omat RP film with an intensifying screen. The film was developed according to the instructions of the manufacturer after 18 to 24 h of exposure at −70°C.

Preparation of monoclonal antibodies. Approximately 2×10^7 Sp 2/0 myeloma cells were fused with 1.6×10^8 spleen cells of the immune mouse by using polyethylene glycol 4000 as a fusogen and previously described methods (6, 10). The fused cells were suspended in hypoxanthine-azaserine selective medium, and approximately 2.5×10^5 cells were distributed in 0.2-ml samples into wells of 96-well tissue culture plates. All plates were placed in a humidified 5% CO_2 incubator at 37°C. At the appropriate time, the supernatant in each well was collected and screened for antibody activity against Ba by an indirect ELISA. Antibody specificities of supernatants from wells that were positive by ELISA were examined by Western blot analysis against Ba and LPS. Cells from wells which showed antibody patterns of interest were then recloned by the limiting dilution method. Supernatants from these monoclones were retested for antibody activities and specificities. Cross-reactivity with heterologous antigens was also determined by indirect ELISA and by Western blot analysis with the heterologous antigens for coating the wells in ELISA plates and in SDS-PAGE by transblotting onto the nitrocellulose strips, respectively. The monoclone which showed specificity only to *S. typhi* (salmonella group D) was expanded to large-scale production of antibodies by culturing in 275-ml tissue culture bottles containing 50 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum. The supernatants containing the specific antibodies were pooled and concentrated with polyethylene glycol 20,000. The concentrated material was passed through a protein A–Sepharose CL-4B column. After the unbound materials were washed with 0.1 M PBS (pH 7.2), the immunoglobulin G was eluted out with 0.1 M glycine hydrochloride buffer (pH 2.8). The pH of the proteins was adjusted to 7.4 by adding 1 M K_2HPO_4 solution, and the protein mixture was dialyzed against distilled water and concentrated with polyethylene glycol 20,000.

Peroxidase-labeled monoclonal antibodies. The concentrated monoclonal antibody specific to antigen 9 of *S. typhi* was labeled with horseradish peroxidase by the two-step glutaraldehyde method described by Voller et al. (23).

Indirect ELISA. An indirect ELISA was used for screening the hybrids and also for measuring the antibody titers of immune mouse sera and monoclonal antibody preparations. Each well in the ELISA plate was sensitized with 100 μl of Ba (10 μg/ml) in PBS (pH 7.4). The plate was incubated overnight at 4°C. The unbound antigens were removed by washing with PBS-Tween 20 (PBS-T). The unbound surface of the wells was blocked with 100 μl of 1% bovine serum albumin in PBS at 37°C for 1 h. All wells were then washed with PBS-T, and 100-μl volumes of mouse antibodies were added to appropriate wells. Control negative wells to which only PBS was added were included in the plate. The antigen-antibody reaction took place at 37°C for 1 h. After the wells were washed, 100 μl of 1:1,000 peroxidase-labeled rabbit anti-mouse immunoglobulins (Dakopatts) was added to each well. After 1 h of incubation, the wells were washed and substrate was added. Substrate conversion proceeded for 30 min at 25°C. The reaction was stopped by adding 50 μl of 1 N NaOH. The OD of each well was measured against blanks (control negative wells to which no antibody was added) at 405 nm. The wells which had ODs equal to or higher than 0.05 were recorded as positive, while the wells with ODs lower than 0.05 were negative (no color change). One indirect ELISA unit was the smallest amount of the antibody which gave a positive reaction.

Double-antibody sandwich ELISA. The double-antibody sandwich modification of the ELISA was used for detecting *S. typhi* antigen in the plasma and urine of patients. Each well of an ELISA plate was coated with 100 μl of 10-μg/ml rabbit anti-Ba immunoglobulins (Ab-1) in PBS. Blocking was done with 1% bovine serum albumin, and then 100 μl of clinical specimens (plasma or urine) was added to the sensitized wells, except for the blank well, to which PBS was added. After incubation and washing of the wells, 100 μl of peroxidase-labeled specific monoclonal antibody (Ab-2) diluted 1:50 was added to each well. The plate was subsequently incubated and washed, and substrate solution was added. The reaction was stopped, and the OD of each well

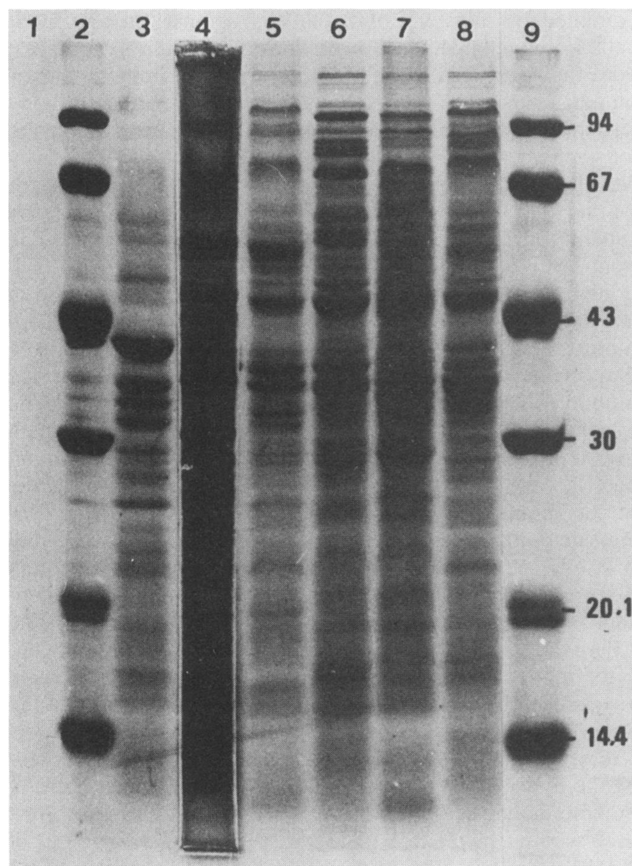

FIG. 1. Protein profiles of various salmonella antigens (Coomassie blue staining). Lanes: 1, TE-LPS of *S. typhi*; 3, Ba of *S. typhi*; 4, WC of *S. typhi*; 5 to 8, WC of *S. panama* and *S. paratyphi* A, B, and C, respectively; 2 and 9, low-molecular-weight standards. Numbers on right show molecular weight (10^3).

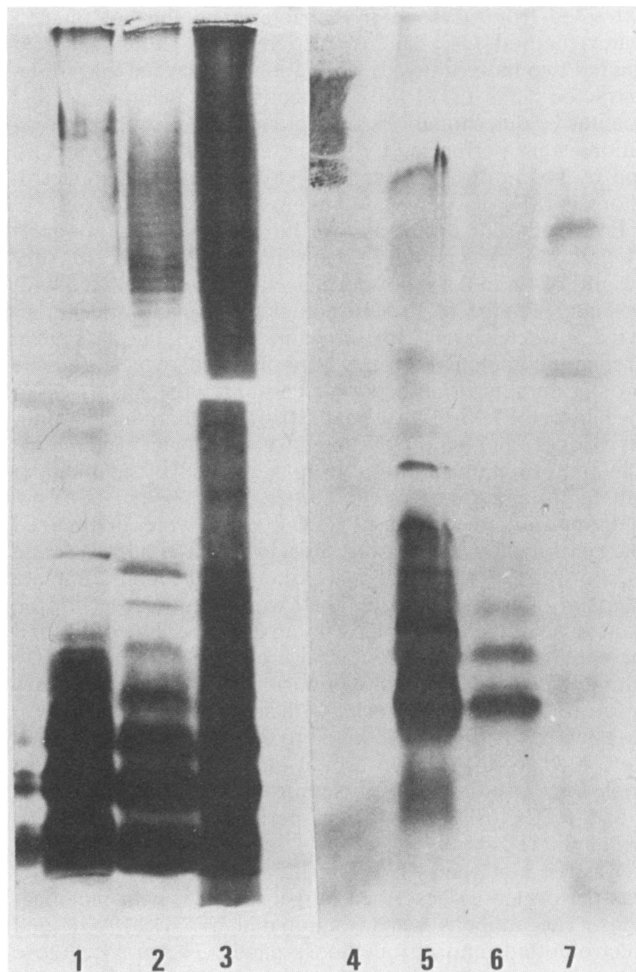

FIG. 2. Profiles of salmonella antigens revealed by SDS-PAGE and silver staining. Lanes: 1, Ba of *S. typhi*; 2, WC of *S. typhi*; 3, TE-LPS of *S. typhi*; 4, WC of *S. panama*; 5 to 7, Ba of *S. paratyphi* A, B, and C, respectively.

was read against a blank with a Uniskan II ELISA reader (Labsystems, Helsinki, Finland).

Triple-antibody ELISA. The initial procedure of the triple-antibody ELISA was essentially the same as that of the double-antibody sandwich ELISA. After the wells were washed to remove the unbound antigens, 16 indirect ELISA units of unlabeled specific monoclonal antibody (Ab-2) was added to each well. After incubation for 1 h at 37°C, the wells were washed and 1:500 peroxidase-labeled rabbit anti-mouse immunoglobulins (Dakopatts) (Ab-3) were added to each well. After incubation and washing of the wells, substrate was added and the reaction proceeded for 30 min before being stopped with 1 N NaOH. The OD of each well was read as for the double-antibody sandwich ELISA.

RESULTS

From 11 g of acetone-dried *S. typhi*, 323 mg of the dried Ba which contained 68.2% proteins was obtained. Profiles of these proteins were revealed by fractionating the Ba in SDS-PAGE and staining with Coomassie blue dye (Fig. 1). The antigen also contained LPS as revealed by silver staining (Fig. 2). From 10 g of acetone-dried *S. typhi* cells, 444 mg (dry weight) of single-extracted LPS was recovered. The single-extracted LPS was reextracted and yielded 127.5 mg of double-extracted LPS with a 0.55% protein content and 52.8 mg of TE-LPS which contained no detectable proteins.

The fusion mixture between 1.6×10^8 immune mouse spleen cells and 2×10^7 Sp 2/0 myeloma cells was distributed into 96-well tissue culture plates. Supernatants from positive wells were screened for antibody specificities by Western blot analysis against Ba. The hybrids from 5 of 129 wells (102, 157, 204, 205, and 250) showing different patterns were selected for recloning. Three monoclones from the five selected wells (157, 204, and 205) reacted with the nonprotein Ba antigen because the antigen did not appear in Coomassie blue staining (Fig. 1) but did appear by silver staining (Fig. 2). The position of this antigen in the SDS-PAGE was known to be the repeating units of the LPS (21).

The monoclonal antibodies produced by monoclones originating from well 102 reacted with the fastest-moving fraction of the Ba in SDS-PAGE. This fraction was located at the position of a complete core polysaccharide (without a repeating unit) in SDS-PAGE (21).

The antibodies produced by monoclones originating from well 250 gave a negative result on Western blot analysis against Ba although they were positive by indirect ELISA. It is possible that the immunoglobulins produced by these clones are immunoglobulin G1 or immunoglobulin M, both of which have a low binding affinity to the staphylococcal protein A (12).

TABLE 1. Percent reactivities of monoclonal antibodies from the representative monoclones against Ba of *S. typhi* and other related organisms by the indirect ELISA[a]

Organism	% Reactivity of monoclone no.[b]:					
	$102B_2$	$157F_9$	$204D_3$	$204F_3$	$205E_4$	$250G_6$
Salmonella typhi 0901	100	100	100	100	100	100
Salmonella typhi[c]	50	112	72.5	72.2	107.4	84.6
Salmonella paratyphi A	118	−0.6	3.2	2.4	8.3	20.6
Salmonella paratyphi B	110	4.8	9.7	0.3	9.9	6.2
Salmonella paratyphi C	38.6	1.7	5.1	2.1	4.9	9.6
Salmonella typhimurium	20.1	2.0	4.1	1.8	1.8	6.7
Salmonella group C_2	38.6	3.8	−1.8	2.1	3.7	15.8
Salmonella group E_4	81	4.5	1.8	1.2	2.1	5.1
Shigella sonnei	0.9	2.7	1.8	2.1	4.9	6.7
Shigella flexneri	8.1	1.0	1.8	1.5	2.1	6.7
Escherichia coli	−1.8	4.5	0.4	0.6	1.8	1.4
Vibrio cholerae	−0.4	1.3	1.8	−0.9	0.3	4.3

[a] The mean percent reactivity of the cutoff value (OD = 0.05) is 19.73.
[b] Percent reactivity was calculated from the formula: (OD of test antigen/OD of *S. typhi* 0901 Ba) × 100.
[c] Pool of antigens from 14 clinical isolates of *S. typhi* was used in the ELISA.

The specificities of the monoclonal antibodies produced by monoclones obtained from wells 102, 157, 204, 205, and 250 were reassessed against Ba of homologous (*S. typhi* 0901 and *S. typhi* from clinical isolates) and heterologous organisms by indirect ELISA. The results (Table 1) showed that the antibodies from clones of wells 157, 204, 205, and 250 were specific only to *S. typhi*, while the antibodies from clones of well 102 cross-reacted with the antigens from all salmonellae tested but did not cross-react with the organisms other than salmonellae. These results and the result of Western blot analysis mentioned above suggest that the well 102 monoclonal antibody is directed to core polysaccharides (or part of them) which are identical in all salmonellae. Only the hybrid clone from well 204 was expanded for further study. The antibody produced by the expanded well 204 clones was retested by indirect ELISA, and a reaction was observed only with *S. typhi* 0901 and all 14 recent clinical isolates of *S. typhi* and *S. panama*, but no cross-reactivity occurred with antigens from *S. paratyphi* A, B, and C, *S. typhimurium*, *Salmonella* groups C_2 and E_4, *Shigella sonnei*, *Shigella flexneri*, *E. coli*, and *V. cholerae*. *S. typhi* possesses O antigens 9 and 12 and *S. panama* contains antigens 1, 9, and 12, and both are group D salmonellae. *S. paratyphi* A and B belong to groups A and B, respectively, and possess O antigens 1, 2, and 12 and 1, 4, 5, and 12, respectively. The monoclonal antibody from clone 204 reacted with *S. typhi* and *S. panama* antigens which share antigens 9 and 12, but did not react with antigens of *S. paratyphi* A or B which contain antigen 12. Thus, the monoclonal antibody is specific to antigen 9 of *Salmonella* LPS. The antibody was found to have the immunoglobulin G3 isotype as assessed by enzyme dot immunoassays against class- and subclass-specific antibodies.

The results of triple-antibody ELISA performed on urine and plasma specimens from the three groups of subjects and normal controls are summarized in Table 2. Normal urine produced no background color or OD (in contrast to that produced by plasma samples). Positive results could be distinguished from negative results by eye. For 43 urine samples from normal individuals, the $\bar{x}$ OD was 0.005 with a standard deviation (SD) of 0.005; thus, the $\bar{x}$ OD + 3 SD was 0.020. When this value was used as a limit between positive and negative reactions, positive tests were found for 11 of 17 urine samples from culture-proven typhoid patients and 12 of 39 urine samples from clinically diagnosed typhoid patients. No false-positive result was observed for all 44 urine samples from normal individuals and urine from three patients with *S. paratyphi* A, *E. coli*, or *Klebsiella* bacteremia, respectively (Table 2). Thus, the specificity and sensitivity of the triple-antibody ELISA performed on urine specimens were 100 and 65%, respectively. The positive and negative predictive values were 100 and 89%, respectively.

When the results of the triple-antibody ELISA performed on urine samples were read by eye or when the OD value at 0.05 was used as a cutoff limit between positive and negative reactions, samples from 8 of 17 culture-proven typhoid

TABLE 2. Detection of *S. typhi* antigen 9 by triple-antibody ELISA in plasma and urine from three groups of patients and normal controls

Specimen from:	Urine					Plasma				
	No. of cases	$\bar{x}$ OD + 3 SD		OD = 0.05		No. of cases	$\bar{x}$ OD + 3 SD		OD = 0.05	
		No. positive	% Positive	No. positive	% Positive		No. positive	% Positive	No. positive	% Positive
Culture-proven typhoid cases	17	11	65	8	47	18	0	0	11	61
Clinically diagnosed typhoid cases	39	12	31	7	18	39	2	5	28	72
Cases with culture-proven paratyphoid A and other gram-negative rods[a]	3	0	0	0	0	3	0	0	3	100
Normal controls	44	0	0	0	0	49	1	2	18	37

[a] *E. coli* and *K. pneumoniae*.

TABLE 3. Detection of *S. typhi* antigen 9 by double-antibody sandwich ELISA in plasma and urine from three groups of patients and negative controls

Specimen from:	Urine			Plasma		
	No. of cases	No. positive	% Positive	No. of cases	No. positive	% Positive
Culture-proven typhoid cases	17	7	41	18	2	11
Clinically diagnosed typhoid cases	39	6	15	39	0	0
Patients with paratyphoid A and *Klebsiella* bacteremia	2	0	0	2	0	0
Negative controls	43	0	0	43	1	2

patients and 7 of 39 clinically diagnosed typhoid patients had positive results (Table 2). The sensitivity was found to decrease from 65 to 47% since specimens from three typhoid patients gave borderline color or ODs which were interpreted as negative. The positive results also decreased from 12 to 7 cases in the second group. However, there was no alteration in the specificity of the assay.

When the TE-LPS was diluted in PBS-T or normal urine, the triple-antibody ELISA could detect as little as 7.8 ng of the antigen. However, the antigen diluted in normal plasma could not be detected because the blank (normal plasma alone without antigen) had a high background OD.

The mean value of the OD detected by the triple-antibody ELISA of 49 plasma samples from normal individuals was 0.048, while the SD was 0.024. This normal background of the test was unexpectedly high and unexplainable. When the $\bar{x}$ OD + 3 SD (0.122) was used as a limit between positive and negative reactions, no positive result was found in 18 culture-proven typhoid cases, while specimens from 2 of 39 clinically diagnosed typhoid patients were considered as positive. A false-positive result was also found in 1 of the 49 plasma samples from the healthy control group (Table 2). However, when the results were judged by looking at the color or OD at 0.05, positive results were found in 11 of 18 typhoid-confirmed cases and in 28 of 39 cases in the second group. False-positive results were observed for samples from all three patients whose blood cultures were positive for *S. paratyphi* A, *E. coli*, and *Klebsiella pneumoniae* and also for 18 of 49 normal control plasma samples. Thus, the specificity and sensitivity of the test performed on plasma samples were 59 and 61%, respectively.

The background OD of double-antibody sandwich ELISA was determined from 43 normal urine samples. The $\bar{x}$ OD was −0.001 and the SD was 0.016. The positive and negative results were then differentiated at an OD of 0.047. Among 17 typhoid-proven cases, 7 (41%) had positive results by these criteria. Among 39 clinically diagnosed typhoid patients, 6 (15%) had positive results. No positive results were found in 43 urine samples from the normal controls and 2 urine samples from patients whose blood cultures were positive for *S. paratyphi* A and *K. pneumoniae* (Table 3). Thus, the specificity and sensitivity of the test performed on urine samples were 100 and 41.1%, respectively.

As the $\bar{x}$ OD + 3 SD of the double-antibody sandwich ELISA of urine samples was 0.047, which was near the OD (0.05) or the limit set by color differentiation, the ELISA judged by either means gave similar results.

Of 18 plasma samples from typhoid-confirmed cases, only 2 were positive by the cutoff $\bar{x}$ OD + 3 SD (0.071). No positive result was obtained from 39 plasma samples from group 2 of the studied subjects and 2 plasma samples from the *S. paratyphi* A and *Klebsiella* bacteremic cases. However, 1 of 49 (2.0%) normal plasma samples gave a positive reaction (Table 3).

The lowest amounts of TE-LPS of *S. typhi* diluted in normal urine and plasma which could be detected by the double-antibody sandwich ELISA were 7.8 and 15.6 ng, respectively.

DISCUSSION

In this study, hybridoma clones producing antibodies specific to core polysaccharides of salmonellae and to antigen 9 of group D salmonellae were constructed. The antibodies obtained from four clones reacted only with *S. typhi* or *S. panama*, which share O antigens 9 and 12, but not with other salmonellae possessing O antigen 12. Thus, the monoclonal antibodies were specific to antigen 9 of group D salmonellae.

Triple-antibody and double-antibody sandwich ELISAs were developed by using monoclonal antibodies produced by clone 204, either as Ab-2 in the former or coupled with peroxidase enzyme and used in the latter for detecting the homologous antigen of *S. typhi* in plasma or urine from typhoid patients. Sensitivity of both assays was determined against purified LPS of *S. typhi* diluted in urine. Both ELISAs could detect as little as 7.8 ng of the antigen in urine specimens, while the double-antibody ELISA detected 15.6 ng of pure LPS in plasma specimens. However, both assays had an unexplainably high nonspecific background when they were tested against normal plasma. Tests of the plasma specimens from the patients were nonspecific. Thus, it was concluded that the two assays should be performed only with the urine specimens. In doing so, the specificity was as high as 100% since no false-positive results were observed in the non-typhoidal bacteremic cases and normal controls examined. This type of high specificity, in fact, has been observed previously with the test for detecting salmonella LPS with rabbit anti-antigen 9 polyclonal antibodies (4, 19). Twelve and six urine samples from 39 patients in group 2 which were positive by triple-antibody ELISA and double-antibody ELISA, respectively, were truly positive.

The antigen 9 in urine from group 1 cases could be detected only in 65 and 41% of the samples by triple-antibody and double-antibody sandwich ELISAs, respectively. The factor which might contribute to the reactivity of these two assays was the monoclonal antibodies used in the systems. High sensitivity of any sandwich assay system clearly depends on the immunological reactivity of the antibodies used. In both assays, the polyclonal antibodies (rabbit anti-Ba immunoglobulins; Ab-1) used for sensitization of the solid phase were potent since the serum was hyperimmune and the titer against Ba was as high as 1:20,480 by the indirect ELISA. The other antibody, the monoclonal antibody (Ab-2) in the triple-antibody ELISA or the peroxidase-conjugated monoclonal antibody in the double-antibody version, had a lower titer than the Ab-1. The monoclonal antibodies were produced from hybridoma clones

grown in in vitro cultures. This method is known to yield only low concentrations of monoclonal antibody. The titer of the antibody preparation was only 1:16 for unconcentrated culture supernatant and 1:64 to 1:512 after concentration (part of the activity was lost after concentration and purification).

The enzyme-conjugated monoclonal antibody used in the double-antibody ELISA was prepared from an affinity-purified concentrated monoclonal antibody which had a titer of 1:512, while an antibody preparation with a titer of 1:64 was used as Ab-2 in the triple-antibody ELISA. Using antibody with a low titer to couple with enzyme conjugate resulted in a weak immunoreactivity. Thus, the sensitivity of the double-antibody sandwich ELISA was low. For the same reason, the sensitivity of the triple-antibody ELISA might be increased by using the more potent Ab-2. Moreover, the low sensitivity of our ELISA may also be due to the single-epitope specificity of the monoclonal antibody (8).

The second factor affecting the sensitivity of these assays is the clinical specimens. These ELISAs were not performed with fresh specimens. Plasma and urine used in this study were kept frozen at −70°C for at least 3 months (the longest time was 13 months) before testing. Moreover, before being transported to the laboratory in Bangkok, most of the specimens had been kept in the refrigerator for some days at the hospital for Khmer refugees in Aranyaprathet. This storage might destroy the antigens and contribute to the growth of contaminating bacteria in both blood and urine. Besides, using the frozen urine samples may not work well in these assays. During the test, it was noticed that some of the frozen urine samples were not homogeneous after being thawed, even at 37°C. Freezing might have caused an uneven distribution of the antigen in the urine or an alteration in the immunological property of the antigen. It is therefore recommended that only fresh urine specimens be used in the assays. The low sensitivity of the two assays in detecting antigen 9 in the urine of confirmed typhoid cases might be due to the intermittent release of the antigen into the specimens. Although it is known that typhoid bacilli are excreted into the urine of the patients during and after bacteremia, this may be intermittent.

Using a mixture of monoclonal antibodies directed against several specific epitopes of *S. typhi* such as anti-Vi or anti-specific protein (Tp antigen or antigen 28) (22) together with anti-antigen 9 monoclonal antibodies in the triple-antibody ELISA is another way to improve the sensitivity of the assay since the monoclonal antibodies will react with different target antigens (epitopes). Therefore, using anti-antigen 9 along with other specific monoclonal antibodies may be superior to using the anti-antigen 9 alone since more antigens which have been captured by the polyclonal antibodies (Ab-1) will be sandwiched by the monoclonal antibodies and subsequently be detected by the anti-mouse immunoglobulin-enzyme conjugate.

The ELISAs developed in this study have several potential advantages. First, they are reliable when tested with urine specimens, which are easy to collect from the patients. Second, the ELISA for typhoid antigen can be performed rapidly (each step takes 1 h) (shorter time intervals have not been studied). Finally, the majority of results performed on urine samples can be monitored by eye (except for some borderline reactions). This is useful for most diagnostic laboratories in developing countries where ELISA readers are not available.

ACKNOWLEDGMENTS

We thank Rob Overtoom and Ben de Chermont, Committee for Coordination of Services to Displaced Persons in Thailand, for their great help in collecting and transporting the clinical specimens. Thanks are extended to David Taylor, Armed Forces Research Institute of Medical Science, Bangkok, Thailand, for his valuable comments. We thank Thanya Nirantasook for typing the manuscript.

The investigation was supported by a research grant authorized by Santasiri Sornmani, Dean of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

LITERATURE CITED

1. **Appassakij, H., N. Banchuin, S. Sarasombath, B. Rungpitarangsri, S. Manatsathit, P. Komolpit, and T. Sukosol.** 1987. Enzyme-linked immunosorbent assay for detection of *Salmonella typhi* protein antigen. J. Clin. Microbiol. **25:**273–277.
2. **Barber, C., I. R. Vladoianu, and G. H. Dimachae.** 1966. Contributions to the study of salmonella: immunological specificity of proteins separated from *Salmonella typhi*. Immunology **11:**287–296.
3. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody in radioiodinated protein A. Anal. Biochem. **112:**195–203.
4. **Carlsson, M., A. Hedin, M. Inganas, B. Harfast, and F. Bromberg.** 1985. Purification of *in vitro* produced mouse monoclonal antibodies. A two-step procedure utilizing cation exchange chromatography and gel filtration. J. Immunol. Methods **75:**89–98.
5. **Edelman, R., and M. M. Levine.** 1986. Summary of an international workshop on typhoid fever. Rev. Infect. Dis. **8:**329–349.
6. **Galfre, G., S. C. Howe, and C. Milstein.** 1977. Antibodies to major histocompatibility antigen produced by hybrid cell lines. Nature (London) **266:**550–552.
7. **Gupta, A. K., and K. M. Rao.** 1979. Simultaneous detection of *Salmonella typhi* antigen and antibody in serum by counterimmunoelectrophoresis for an early and rapid diagnosis of typhoid fever. J. Immunol. Methods **30:**349–353.
8. **Hewitt, J., A. R. M. Coates, D. A. Mitchison, and J. Ivanyi.** 1982. The use of murine monoclonal antibodies without purification of antigens in the serodiagnosis of tuberculosis. J. Immunol. Methods **55:**205–211.
9. **John, T. J., K. Sivadasan, and B. Kurein.** 1984. Evaluation of passive bacterial agglutination for the diagnosis of typhoid fever. J. Clin. Microbiol. **20:**751–753.
10. **Khusmith, S., S. Tharavanij, J. Patarapotikul, and D. Koonrangsrisomboon.** 1984. Characterization of monoclonal antibodies against blood forms of *Plasmodium falciparum* by the immunofluorescent test. Asian Pac. J. Allergy Immunol. **2:**91–95.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
12. **Lindmark, R., K. Thoren-Tolling, and J. Sjoquist.** 1983. Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. J. Immunol. Methods **62:**1–13.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
14. **Paus, E., O. Bormer, and K. Nustad.** 1982. Radioiodination of proteins with the iodogen method, p. 161–171. *In* Radioimmunoassay and related procedures in medicine 1982. Proceedings of a Symposium, Vienna, Austria. IAEA, Austria.
15. **Rockhill, R. C., L. W. Rumans, and M. Lesmana.** 1980. Detection of *Salmonella typhi* D, Vi, and d antigens by slide coagglutination in urine from patients with typhoid fever. J. Clin. Microbiol. **11:**213–216.
16. **Rockhill, R. C., L. W. Rumans, and M. Lesmana.** 1981. Slide coagglutination for *Salmonella typhi* antigens in broths inoculated with feces from typhoid patients. Southeast Asian J. Trop. Med. Public Health **12:**528–532.

17. **Shetty, N. P., H. Srinivasa, and P. Bhat.** 1985. Coagglutination and counter-immunoelectrophoresis in the rapid diagnosis of typhoid fever. Am. J. Clin. Pathol. **84:**80–84.
18. **Sundararaj, T., B. Ilango, and S. Subramanian.** 1983. A study on the usefulness of counter-immunoelectrophoresis for the detection of *Salmonella typhi* antigen in the sera of suspected cases of enteric fever. Trans. R. Soc. Trop. Med. Hyg. **77:**194–197.
19. **Svenungsson, B., H. Jorbeck, and A. A. Lindberg.** 1979. Diagnosis of salmonella infections: specificity of indirect immunofluorescence for rapid identification of salmonella enteritis and usefulness of enzyme-linked immunosorbent assay. J. Infect. Dis. **140:**927–936.
20. **Taylor, D. N., J. R. Harris, T. J. Barrette, N. T. Hargrett, I. Prentzal, C. Valdivieso, C. Palomino, and M. M. Levine.** 1983. Detection of urinary Vi antigen as a diagnostic test for typhoid fever. J. Clin. Microbiol. **18:**872–876.
21. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
22. **Tsang, R. S., P. Y. Chau, S. K. Lam, J. T. Brooy, and D. Rowley.** 1981. Antibody response to the lipopolysaccharide and protein antigens of *Salmonella typhi* during the typhoid infection. I. Measurement of serum antibodies by radioimmunoassay. Clin. Exp. Immunol. **46:**508–514.
23. **Voller, A., D. Bidwell, and A. Bartlett.** 1980. Enzyme-linked immunosorbent assay, p. 359–371. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. American Society for Microbiology, Washington, D.C.
24. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide: extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5:**83–91.