

## Serodiagnosis of Typhoid Fever by Enzyme-Linked Immunosorbent Assay Determination of Anti-*Salmonella typhi* Lipopolysaccharide Antibodies

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Serum samples from 22 patients with proven typhoid fever, 60 febrile nontyphoidal patients, and 120 healthy subjects were tested for immunoglobulin A (IgA), IgG, and IgM anti-*Salmonella typhi* lipopolysaccharide antibodies by an enzyme-linked immunosorbent assay. The levels of all three classes of immunoglobulin anti-lipopolysaccharide were higher in typhoid patients than in controls; the test for IgM anti-lipopolysaccharide gave the best discrimination between typhoid and nontyphoidal sera. The absorbance values obtained with the enzyme-linked immunosorbent assay for IgM anti-lipopolysaccharide were highly correlated to the titers of anti-O agglutinins. However, the enzyme-linked immunosorbent assay was much more specific than the Widal test, and hence it could be a useful tool for the serological diagnosis of typhoid fever with a single blood sample.

A definite diagnosis of typhoid fever can only be obtained by the presence of *Salmonella typhi* in the blood or in other biological fluids. However, blood cultures, which are positive in 80% of typhoid patients during the first week of fever, show decreasing positive results thereafter (6). In addition, bacteriological cultures may be useless because of ongoing antibiotic treatment. The validity of the Widal agglutination test, the most common test for the serological diagnosis of typhoid fever, has been questioned (2, 4). Agglutinins are frequently found in normal and febrile nontyphoidal sera, particularly in endemic areas, so that is practically impossible to define a diagnostic titer for a single Widal test (5).

Recently, Tsang et al. (7) reported that radioimmunoassay determination of antibodies to the lipopolysaccharide (LPS) or protein antigens of *S. typhi* is more specific and sensitive than the Widal test for the diagnosis of typhoid fever in endemic areas. In this study we tested sera from typhoid patients and from several febrile and nonfebrile controls for immunoglobulin A (IgA), IgG, and IgM anti-LPS antibodies by an enzyme-linked immunosorbent assay (ELISA). We also compared the diagnostic usefulness of the ELISA with that of the Widal test.

### MATERIALS AND METHODS

**Sera.** Twenty-nine serum samples were collected from 22 patients with typhoid fever diagnosed by blood cultures positive for *S. typhi*. One or two samples were obtained from each patient during a period ranging from 3 to 21 days after the onset of fever. An additional blood sample was taken from seven patients 1 to 3 months later. Control serum samples were from 60 patients (27 males, age range of 4 to 83 years) suffering from febrile illnesses other than typhoid fever: cholecystitis and cholangitis (eight cases), urinary tract infection (eight cases), solid neoplasm (seven cases), acute brucellosis (five cases), bacterial or viral acute pneumonia (four cases), upper respiratory infection (four cases), gastrointestinal infection (four cases), pulmonary tuberculosis (four cases), lupus erythematosus systemic (three cases), infectious mononucleosis (two cases), ulcerative colitis (two

cases), infective endocarditis (two cases), Hodgkin's lymphoma (two cases), enteric fever (*Salmonella paratyphi* B, two cases), malaria (one case), visceral leishmaniasis (one case), and Guillain-Barré syndrome (one case). Diagnosis was made by isolation of the etiological agent(s), by laboratory, instrumental, and clinical findings, or by both. Additional control serum samples were from 120 healthy subjects (68 males, age range of 5 to 83 years). Fourteen control subjects had had typhoid fever 7 to 25 years before, and 27 had received TAB vaccine 2 to 30 years before.

**ELISA.** Optimal conditions and reproducibility of the assay are as previously reported (3). LPS W of *Salmonella typhosa* 0901 (Difco Laboratories, Detroit, Mich.) was used at 5 µg/ml in 0.05 M carbonate buffer (pH 9.6) to coat the wells of polystyrene microtiter plates (Dynatech M129 A) by overnight incubation. After three washings with phosphate-buffered saline containing 0.05% Tween 20 (phosphate-buffered saline-Tween), plates were incubated for 2 h with serum samples diluted 1:200 in phosphate-buffered saline-Tween supplemented with 4% bovine serum albumin, washed again, and then filled with class-specific (anti-IgA, anti-IgG, anti-IgM) goat anti-human immunoglobulins conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.), diluted 1:1000 with phosphate-buffered saline-Tween containing 1% bovine serum albumin. After 2 h of incubation, nonreactive conjugate was washed away, and *p*-nitrophenylphosphate, 1 mg/ml in 0.05 M carbonate buffer (pH 9.8) containing 0.001 M MgCl<sub>2</sub>, was added. The reaction of the substrate was stopped after 30 min with 50 µl of 3 M NaOH. The assay was performed at room temperature, with 200 µl of each reactive, in quadruplicate wells. Absorbance at 405 nm was read against a blank (substrate in buffer) on a Eppendorf photometer after transferring the contents of duplicate wells into polystyrene cuvettes. Results were expressed as absorbance values at one dilution of the test sample: optical readings at a 1/200 dilution were in fact directly correlated to titers (3). Absorbance values of the samples were corrected by reference to a positive control serum sample from a typhoid patient (Widal anti-O titer = 1:160), which was given the mean absorbance value of 20 replicate assays performed on different days (coefficient of

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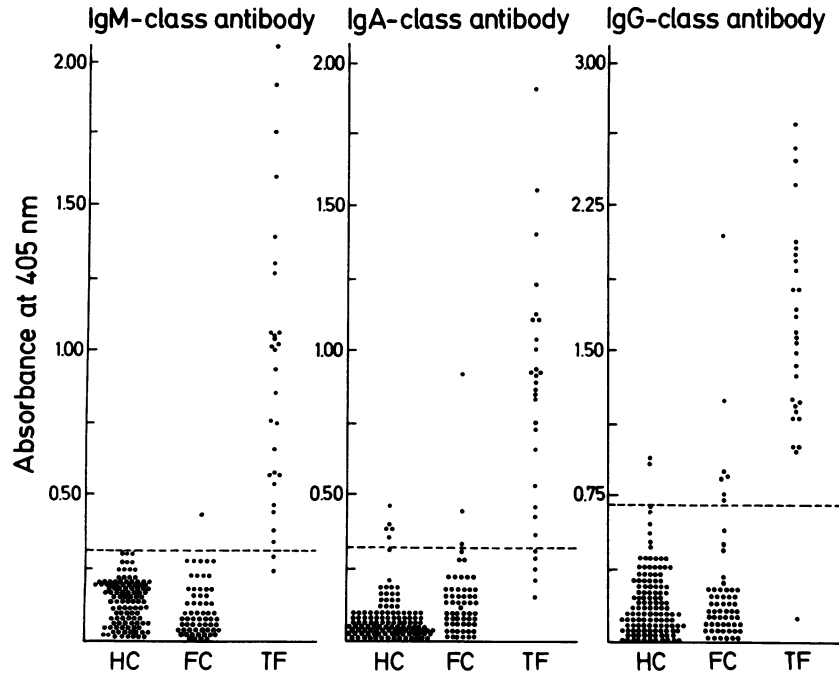


FIG. 1. Immunoglobulin anti-*S. typhi* LPS: ELISA absorbance values for serum samples from healthy controls (HC), febrile nontyphoidal patients (FC), and patients with typhoid fever (TF). The dashed lines represent the cut-off value (mean + 3 standard deviations of the values for healthy controls).

variation: 5.4% for IgA, 5.9% for IgG, 5.0% for IgM). The reference sample was included in each plate tested. Corrected sample values were calculated according to the following formula: (measured absorbance of sample × given absorbance of positive control)/measured absorbance of positive control.

**Widal test.** Titration of anti-*S. typhi* O agglutinins was performed by the standard tube method, with antigen suspensions from Sclavo (Siena, Italy) and twofold serum dilutions starting from 1:40.

**Rheumatoid factor.** The rheumatoid factor was detected by reacting the sera with a suspension of 1% Latex particles sensitized with human gamma globulins (Rheuma test; Sclavo).

**RESULTS**

The absorbance values obtained with sera from normal, typhoidal, and febrile nontyphoidal subjects in the test for IgM, IgG, and IgA anti-LPS of *S. typhi* are reported in Fig. 1. There was a clear distinction between IgM ELISA values for sera from subjects with typhoid and those for sera from both normal and febrile controls. Two serum samples from two patients with typhoid, obtained early in the course of the

disease (days 3 and 6 after the onset), had IgM anti-LPS levels within the range of normal controls. One of these two patients showed levels above the normal range at the second sample, obtained 7 days later; no additional sample was available from the other patient. Only one serum sample from a febrile nontyphoidal subject had an elevated IgM reading. This serum sample was from a woman with acute bacterial pneumonia and a positive agglutination test (1:40).

The rheumatoid factor was detected in 14 of the 60 febrile control serum samples; the rheumatoid factor did not appear to interfere with the IgM anti-LPS test. Only two typhoid patients had serum samples positive for the rheumatoid factor.

A wide range of IgG anti-LPS ELISA values, including some high readings, were found for healthy controls. However, all but one serum sample from patients with typhoid had IgG anti-LPS readings higher than the upper limit for normal controls. Elevated IgG readings were also observed with serum samples from many febrile nontyphoidal patients, two of them showing values above 1.00.

IgA ELISA values for serum samples from patients with typhoid were clearly elevated; however, there was also a minor overlap with those of normal and febrile controls.

TABLE 1. Immunoglobulin anti-*S. typhi* LPS mean ELISA absorbance values for healthy and febrile nontyphoidal controls

Serum source	Previous vaccination, typhoid fever, or both	No. of subjects	Immunoglobulin anti-LPS ELISA values (mean ± SD) for:		
			IgM	IgA	IgG
Healthy controls	Yes	21	0.10 ± 0.07	0.08 ± 0.10	0.23 ± 0.22
	No	45	0.13 ± 0.16	0.08 ± 0.08	0.19 ± 0.18
Febrile controls	Yes	20	0.11 ± 0.09	0.12 ± 0.09	0.27 ± 0.30
	No	31	0.11 ± 0.10	0.12 ± 0.10	0.23 ± 0.22

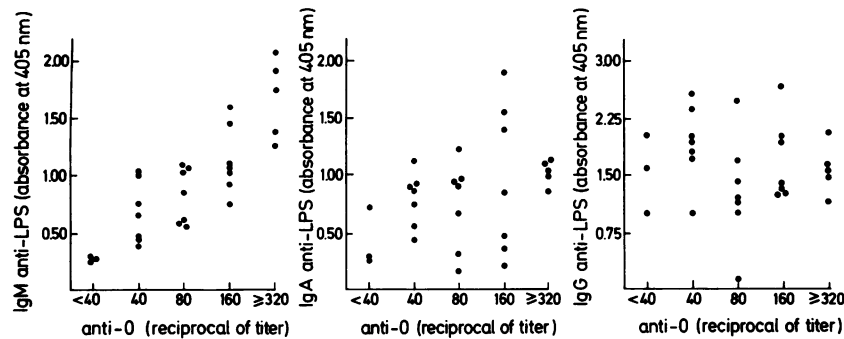


FIG. 2. Immunoglobulin anti-*S. typhi* LPS: correlation between ELISA absorbance values and titers of anti-O agglutinins for serum samples from patients with typhoid fever.

The mean immunoglobulin anti-LPS readings for sera from control subjects who had been previously vaccinated or had had typhoid fever, or both, and for sera from "clean" controls are reported in Table 1. IgG anti-LPS readings were slightly higher for subjects who had been vaccinated or had had typhoid fever, or both, but the difference was not significant.

The correlation between absorbance values of the immunoglobulin anti-LPS test and titers of anti-O agglutinins in serum samples from patients with typhoid is illustrated in Fig. 2. The Widal test was highly correlated to ELISA IgM values ( $r = 0.874$ ;  $P < 0.001$  by the Spearman rank correlation test) and, to a lesser extent, to IgA readings ( $r = 0.385$ ;  $P < 0.05$ ), but not to IgG readings ( $r = 0.026$ ).

Immunoglobulin anti-LPS absorbance values for the 29 serum samples from typhoid patients in the acute phase and

for the additional 7 serum samples obtained during the convalescence period are shown in Fig. 3, subdivided according to the time after the onset of typhoid fever. IgM antibody levels peaked by weeks 2 to 3, whereas IgA and IgG levels were similarly elevated in the early and in a more advanced phase of the disease. After 45 to 90 days from the acute phase, elevated IgG values were still observed, whereas IgM and especially IgA levels tended to be normal.

For diagnosis with a single-serum specimen, a cut-off value between negative and positive serum samples in the test ELISA was defined. The means  $\pm$  standard deviation of IgA and IgM readings for healthy subjects were  $0.07 \pm 0.08$  and  $0.11 \pm 0.07$ , respectively. With the mean + 3 standard deviations as the cut-off value, serum samples with an optical density of  $>0.32$  were defined as positive for IgA anti-LPS and serum samples with an optical density of  $>0.31$

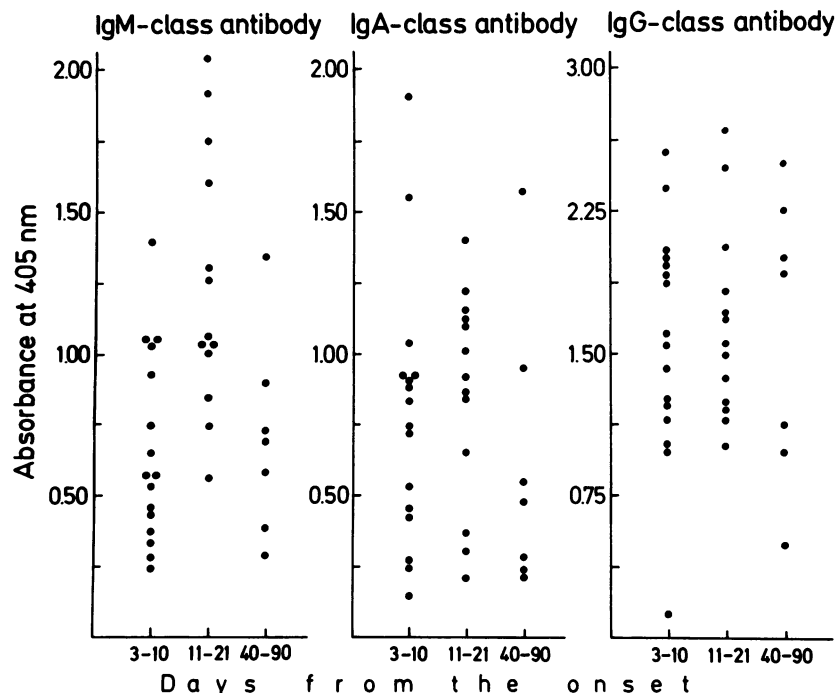


FIG. 3. Immunoglobulin anti-*S. typhi* LPS: ELISA absorbance values for serum samples from patients with typhoid fever, subdivided according to the interval of time after the onset of the disease.

TABLE 2. Results of the ELISA for immunoglobulin anti-*S. typhi* LPS and the Widal test for anti-O agglutinins with typhoid and control serum samples

Serum source	No. of serum samples	No. (%) of serum samples positive for immunoglobulin anti-LPS with:			No. (%) of serum samples with an anti-O titer of:		
		IgM	IgA	IgG	≥1:40	≥1:80	≥1:160
Typhoid patients	29	27 (93.1)	24 (82.7)	28 (96.6)	26 (89.6)	19 (65.5)	12 (41.3)
Febrile controls	60	1 (1.6)	3 (5.0)	7 (11.7)	9 (15.0)	4 (6.6)	1 (1.6)
Healthy controls	120	0	5 (4.1)	2 (1.6)	5 (4.1)	2 (1.6)	0

were defined as positive for IgM anti-LPS. The mean  $\pm$  standard deviation IgG ELISA value for normal controls was  $0.19 \pm 0.17$ ; IgG values of  $>0.70$  were considered as positive.

The diagnostic efficacy of the ELISA and the Widal test are compared in Table 2. Tests for IgG and IgM anti-LPS were both highly sensitive for the diagnosis of typhoid fever, but the IgM test was more specific. The IgA test was less sensitive and less specific than the IgM test. Positivity for both IgM and IgG anti-LPS was present in 26 of 29 serum samples from subjects with typhoid (89.6%) but in none of the control serum samples. The efficacy of the Widal test varied according to the level of the titer chosen as diagnostic. With a titer of 1:40, sensitivity was similar to that of the IgM ELISA test, but specificity was much lower. With a titer of 1:160, the Widal test was as specific as ELISA, but the sensitivity at this dilution was very low.

#### DISCUSSION

Using an ELISA technique, we found that the humoral response to LPS of *S. typhi* in typhoid fever involves all the three major classes of immunoglobulins. These results are in agreement with those obtained by Tsang et al. using a radioimmunoassay method (7).

For all three classes of immunoglobulin anti-LPS, ELISA readings were distinctly higher for typhoid patients than for a series of control subjects. When both sensitivity and specificity were considered, the IgM anti-LPS test gave the best discrimination between typhoid patients and controls. IgM and IgA anti-LPS absorbance values above the cut-off value were observed in a high percentage of serum samples from patients in the early phase of typhoid; however, a higher percentage of serum samples from patients in the early phase of typhoid showed elevated IgG anti-LPS readings. The frequent contact with common *Salmonella* partigens in our geographical area, endemic for salmonellosis, may explain the precocious IgG response in our typhoid patients. On the other hand, a late IgG anti-LPS response has been observed by other authors in a nonendemic area (1).

The data of this paper confirm the high prevalence of anti-O agglutinins in endemic areas, especially in subjects with febrile diseases (9 of 60 with a titer of at least 1:40 in this series). The nature and the class of these agglutinins are not known. However, only one of the febrile control subjects with anti-O agglutinins had IgM anti-LPS readings higher than the cut-off value; whereas five had elevated IgG readings (associated with elevated IgA in two subjects), and the remaining three had low anti-LPS readings for all the three immunoglobulin classes. In addition, two of the five healthy subjects with a positive Widal test had elevated IgG anti-LPS readings. These data suggest that agglutinins responsible for a false-positive Widal test may not be specific for LPS, or, if anti-LPS specific, they may belong to a class of immunoglobulins other than IgM, and hence they may be

differentiated from typhoid agglutinins by testing for subclasses of anti-LPS antibodies.

It is important to know the specificity of the ELISA for the typhi group of *Salmonella* organisms. The best way to accomplish this is to test sera from patients with bacteremia due to nontyphoid strains, since these would be most likely to induce a heterotypic immune response. Neither serum sample from the two febrile control patients affected by *S. paratyphi* B infection was positive with ELISA. However, no definite conclusions may be drawn from these findings, and experience with many more patients with bacteremia from nontyphoid strains of *Salmonella* must be reported before one can feel confident of the specificity of the current test.

This assay could be very useful to epidemiologists in investigating outbreaks and in determining whether typhoid fever is occurring in certain less-developed areas, where bacteriological techniques and ELISA methodology are unavailable. One need only collect a single serum specimen from febrile patients, which could be tested for *S. typhi* antibody by IgM ELISA at a later date in an equipped laboratory. As far as developed countries are concerned, there is a potential for the use of ELISA to confirm the diagnosis of typhoid fever. ELISA has some advantages over aggressive culturing techniques (multiple blood cultures, bile culture by string capsule device, bone marrow culture) in that it causes no patient discomfort and it produces faster results.

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