Systemic and Intestinal Immunities after Natural Typhoid Infection

SUTTIPANT SARASOMBATH,^{1*} NAPATAWN BANCHUIN,¹ TASSANEE SUKOSOL,¹ BENJAWAN RUNGPITARANGSI,² AND SATHAPORN MANASATIT³

Departments of Microbiology¹ and Pathology,² Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, and Bamrasnaradura Infectious Disease Hospital, Nonthaburi,³ Thailand

Received 6 October 1986/Accepted 24 February 1987

A 2-year study of systemic and intestinal immunity to *Salmonella typhi* was performed in 14 patients who were suffering from typhoid fever in an attempt to extrapolate the mechanism of immune responses in this disease. The methods employed were the leukocyte migration inhibition agarose test for the measurement of systemic cell-mediated immunity. The systemic immunoglobulin G (IgG) and IgM that were specific to *S. typhi* and anti-O and anti-H agglutinins were measured by indirect enzyme-linked immunosorbent assay (ELISA) and the Widal test, respectively. The immunobead ELISA was used to measure total secretory IgA (SIgA), and indirect ELISA was used to measure SIgA that was specific to *S. typhi* in the intestinal lavage fluid. The results revealed that the patients developed various types of immune response to *S. typhi* that had various magnitudes and durations. After the onset of illness, the cell-mediated immunity persisted for 16 weeks; IgG, IgM, and anti-O and anti-H agglutinin persisted for 2 years, 16 weeks, 16 weeks, and 36 weeks, respectively. SIgA can persist in the gut for about 48 weeks. Thus, the immunities as a whole can barely persist beyond 1 year after the onset of illness, unless there are persistent booster stimulations by *S. typhi* bacilli that exist in the environment, and then the immunities may be lifelong.

Separate studies of systemic and intestinal immunity in patients with typhoid fever have been done by several workers. They have shown the development of cell-mediated immunity and its possible protective role in the disease (15, 20). The systemic antibodies have been identified but their role in protection remains elusive (9, 14). The increment of intestinal antibody response to antigens of *Salmonella typhi* in patients has been shown, but the mechanism of protection has not been well clarified (3, 10).

In this report we describe simultaneous longitudinal studies of systemic antibodies and the cell-mediated immune response (CMIR) and intestinal antibody response in patients with typhoid fever in an attempt to extrapolate the immune response mechanism in this disease.

MATERIALS AND METHODS

Subjects. There were 14 subjects that participated in this study, consisting of 12 males and 2 females (age, between 17 and 28 years) with clinically diagnosed typhoid fever. All cases of typhoid fever were confirmed by positive hemoculture for *S. typhi*. Informed consent for participation in this study was obtained from each patient. The guidelines established by Mahidol University for human experimentation were followed.

The controls consisted of 55 healthy adult males (age, between 19 and 21 years). These controls had never had clinically apparent typhoid or paratyphoid fever and had not received typhoid or paratyphoid vaccinations for at least 7 years prior to this study. They also had minimal or no anti-O or anti-H agglutinins and minimal or no specific CMIR to the antigen of *S. typhi*, as determined by the leukocyte migration inhibition agarose test (LMIT).

Sample collections. Blood and intestinal lavage specimens were collected at the same time from each subject. The samples were collected only once from the controls but were

collected from the patients at 4, 8, 12, 16, 20, 24, 36, and 48 weeks and 1.5 and 2 years after the onset of illness.

The serum samples were divided into fractions and stored at -20°C until use. The lymphocytes that were obtained from heparinized blood were processed and examined for CMIR within 1 h after collection. Intestinal lavage specimens were collected successfully from 8 male patients and 49 controls. The method of collection was as described by Sack et al. (17), with the use of slight modifications described previously (3). Briefly, the subjects were asked to drink a large volume (approximately 3 liters) of an isotonic balanced salt solution which contained no nutrients; this resulted in a brief attack of diarrhea. Specimens of approximately 500 ml of watery stool were collected from each patient over a period of 2 to 3 h. Each specimen was inactivated immediately in a water bath at 56°C for 15 min and then filtered through gauze and chilled in an ice bath. The filtrates were then centrifuged at $10,000 \times g$ for 10 min, filtered through a membrane (pore size, $0.45 \mu\text{m}$; Millipore Corp., Bedford, Mass.), and concentrated approximately 25-fold by ultrafiltration through dialysis tubing under vacuum. The concentrated specimens were divided into 0.5-ml fractions and stored at -70°C until use.

Preparation of antigen. The protein (Bp) antigen from *S. typhi* 0-901 was used in this study, because the same type of preparation from *S. enteritidis* and *S. typhimurium* was believed to contain some protective antigen, as has been described previously by Barber and Eylar in a mouse model (6, 7). The method of preparation was as described by Barber and co-workers (5, 8) with slight modifications. Briefly, washed and acetone-dried bacterial cells were extracted with Veronal buffer (pH 8.4; Winthrop Laboratories, Div. Sterling Drug Co., New York, N.Y.), and the protein was precipitated out of the extract with trichloroacetic acid. The precipitate was washed, resolubilized, lyophilized, and stored at 4°C until use. The protein content was assayed by the method described by Lowry and as modified by Hartree (13). This antigen had a small amount of lipopolysaccharide

* Corresponding author.

contamination (21), but no Vi antigen was present because *S. typhi* 0-901 lacked this particular antigen.

Assessment of CMIR. The systemic cell-mediated immunity to *S. typhi* was assessed by LMIT as described previously (19). The optimum concentration of Bp antigen for use in the LMIT was determined by checkerboard titration; it was determined to be 125 $\mu\text{g/ml}$. The results were expressed as the leukocyte migration inhibition (LMI) index. The nonparametric Mann-Whitney U test was employed for the evaluation of the statistical significance of differences in this part of study.

Assessment of the systemic antibody response. Antibodies to the O and H antigens of *S. typhi* were determined in all serum samples by the standard Widal agglutination test by using suspensions of organisms that were obtained from Gamma Diagnostics (Div. Gamma Biologicals, Houston, Tex.).

The systemic immunoglobulin G (IgG) and IgM specific to *S. typhi* were determined by an indirect enzyme-linked immunosorbent assay (ELISA) by a previously described technique (4), which was as follows. The Bp antigen was diluted with 0.05 M carbonate buffer (pH 9.8) to an optimum concentration of 5 $\mu\text{g/ml}$ and was coated onto each well of MicroELISA Immulon plates (Dynatech Produkta, Kloten, Switzerland) by incubating for 3 h at 37°C. The plates were washed 3 times with 0.01 M phosphate-buffered saline (pH 7.1) containing 0.15% (vol/vol) Tween 20 (PBST) and tapped dry. Then, serial fivefold dilutions or reference serum samples were added and incubated at 4°C overnight. Each specimen was assayed in duplicate. After three washes with PBST, alkaline phosphatase-conjugated goat anti-human IgG or IgM (Southern Biotechnology, Birmingham, Ala.) was added to each well. The plates were incubated at 30°C overnight. After excess conjugate was washed out 3 times with PBST, freshly prepared *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) was added at 1 mg/ml to each well. After 45 min at 30°C, the reactions were terminated by addition of 3 M NaOH. The A_{405} of the color that developed was measured immediately with a spectrophotometer (Titertek Multiskan; Flow Laboratories, Bonn, Federal Republic of Germany).

In addition to the reference serum sample, a direct conjugate control and a substrate blank were included in each plate. PBST was substituted for the specimen in the direct conjugate control and for both specimen and the conjugate in the substrate blank. The wells for the direct conjugate control provided a control for the extent of nonspecific conjugate binding, and the wells for the substrate blank provided a control for spontaneous hydrolysis of the substrate. To calculate the results, the mean absorbance of each dilution sample minus the absorbance of the direct conjugate control, which was performed in the same plate, was used.

For the quantitation of IgG and IgM specific to *S. typhi*, a curve of a reference serum sample was constructed by plotting absorbance units against corresponding dilutions. The endpoint of the reaction was read at a point of 0.4 absorbance unit above the base line, and the corresponding dilution which gave this endpoint reading was determined from the curve. For unknown specimens, curves were also constructed, and endpoint dilutions were determined in the same manner. Because there were no recognized units for anti-Bp antibodies, the reference serum sample was arbitrarily assigned a value of 100 units. Thus, the titers for unknown specimens could be quantitated in terms of arbitrary antibody units (aau) relative to that for the reference serum sample.

Assessment of intestinal antibody response. The secretory IgA (SIgA) specific to *S. typhi* in the intestinal lavage specimens was determined by indirect ELISA in the same manner described above by using a 5- $\mu\text{g/ml}$ concentration of Bp antigen. The results are expressed in aau/1 mg of total intestinal SIgA of that specimen.

The total intestinal SIgA level was determined by immunobead ELISA by using rabbit anti-human IgA that was covalently bonded to polyacrylamide beads (Bio-Rad Laboratories, Glattburg, Switzerland), by the method described by Sack and co-workers (18). The standard SIgA that was used was obtained from pooled milk from healthy Thai women; the milk contained 0.75 mg of SIgA per ml. The results are expressed as milligrams of total SIgA in 1 ml of lavage fluid.

RESULTS

CMIR. By the LMIT the mean $\pm$ standard error of the mean (SEM) of the LMI index, which was determined in 55 healthy controls, was 0.83 ± 0.02 . A significant difference in the LMI index between the patients and the healthy controls was noted at 4 weeks ($P < 0.01$), 8 weeks ($P < 0.01$), and 16 weeks ($P < 0.01$) after the onset of illness by the nonparametric Mann-Whitney U test. Thus, the positive CMIR in the patients was irregular and persisted for only 16 weeks after the onset of illness (Fig. 1).

Systemic antibody response. The titers of the O and H agglutinins and the geometric means are shown in Fig. 2 and 3. The peaks of both agglutinins were observed at week 4 after the onset of illness, but it declined rapidly thereafter. The geometric means of O and H agglutinins from 55 healthy controls were below 80 and 100, respectively; therefore, the significant level of both antibodies ($O > 80$, $H > 100$) persisted for only 16 weeks after the onset of illness for anti-O agglutinin and 36 weeks for anti-H agglutinin.

The responses of specific IgG and IgM to Bp antigen are presented in Fig. 4 and 5. The means $\pm$ SEM of IgG and IgM in the controls were 30.7 ± 4.2 and 14.4 ± 2.4 aau, respectively. An increment of both antibodies was observed, and the peak was seen at week 4. The mean IgG level was higher than that of IgM, and the former antibody persisted longer (2 years for IgG, 16 weeks for IgM).

Intestinal antibody response. The result of specific SIgA to Bp antigen is shown in Fig. 6. The mean $\pm$ SEM of this antibody in the 49 controls was 55.3 ± 16.6 aau/1 mg of total SIgA. The elevation of this antibody in patients with typhoid fever was observed through 48 weeks after the onset of illness; the peak was seen at week 4.

DISCUSSION

To extrapolate the mechanism of immune responses in patients with typhoid fever, we designed simultaneous longitudinal studies of systemic and intestinal immune responses in patients suffering from this disease. The length of the study was 2 years; only 43% (6 of 14) of the patients could be followed through the entire period; the remainder of them, however, withdrew from the study intentionally or were lost to follow-up. All patients recovered fully without any complications within 2 weeks after the appropriate antibiotic therapy. All were in good health during the entire follow-up period. Unfortunately, we could not obtain the data earlier than week 4 after the onset of illness, because all patients came to the hospital late in course of the disease, usually after they had tried to treat themselves by purchasing medicine from local drugstores.

FIG. 1. Specific CMIR to Bp antigen in the patients as determined by LMIT; the means $\pm$ SEM of the LMI index are shown. The broken line represents the mean value for 55 controls.

The results of our study reveal that significant cell-mediated immunity, as demonstrated by LMIT, develops during the course of disease until 16 weeks after the onset of illness. The protein (Bp) antigen, which was extracted from *S. typhi* for use in this study, is believed to contain some protective antigen, according to the results of studies on mouse typhoid fever described by Barber and Eylan (6, 7). Thus, the positivity of LMIT to the Bp antigen in the patients should indicate the immune response to some protective antigen of *S. typhi* by the antigen-specific T lymphocytes and

monocytes and macrophages. The protective role of cell-mediated immunity in mouse (11, 16) and human (15, 20) typhoid fever has been emphasized.

The development of anti-O and anti-H agglutinins during the course of disease and recovery phase indicate the antibody responses to the lipopolysaccharide and the flagella of *S. typhi*, respectively. Although several investigators have shown that these antibodies have some protective effect in mouse typhoid fever (12), the opposite was observed in human typhoid fever (9, 20). However, no defini-

FIG. 2. Anti-O agglutinin in the patients. The reciprocal titer (●) of this antibody from each patient and the geometric means of the titer (x) from samples obtained at the indicated times are shown.

FIG. 3. Anti-H agglutinin in the patients. The reciprocal titer (●) of this antibody from each patient and the geometric means of the titer (×) from samples obtained at the indicated times are shown.

FIG. 4. Specific systemic IgG response to Bp antigen in the patients; the amounts (in aau; means $\pm$ SEM) of this antibody at the different sampling times are shown. The broken line represents the mean level of this antibody in 55 controls.

FIG. 5. Specific systemic IgM response to Bp antigen in the patients; the amounts (in aau; means $\pm$ SEM) of this antibody at the different sampling times are shown. The broken line represents the mean level of this antibody in 55 controls.

tive study has been done to show the protective role of anti-O and anti-H agglutinins in humans. It was noticed that the anti-H agglutinin persisted longer than the anti-O agglutinin. This was to be expected because anti-O agglutinin is well known to be composed mainly of IgM, which has a shorter life than IgG, which is the major immunoglobulin of anti-H agglutinin. The systemic IgM and IgG response that was specific to the Bp antigen also developed in the patients.

The role of both antibodies in protective immunity also has not yet been clarified.

The intestinal SIgA antibody should play a major role in preventing typhoid fever, because its function is to react with pathogens and inhibit their attachment to the epithelial surface of the gut (1, 2), thus preventing the systemic involvement of the disease. The presence of a significant amount of intestinal SIgA that was specific to the Bp antigen

FIG. 6. Specific SIgA response to Bp antigen in intestinal lavage fluid of the patients; the amounts of this antibody (in aau/1 mg of total SIgA; means $\pm$ SEM) at the different sampling times are shown. The broken line represents the mean level of this antibody in 49 controls.

in this study should indicate the development during the course of typhoid fever of protective intestinal antibody, which is beneficial for prevention of the disease. Apart from SIgA, we have reported the presence of a significant amount of intestinal secretory IgM that is specific to the Bp antigen in these patients during the course of the disease; this antibody is believed to play the same role as SIgA in *S. typhi* infection (11).

In conclusion, the results of this study indicate that patients with typhoid fever develop both systemic and intestinal immunities during the course of disease and recovery period. From these data it can be concluded that the immunities can barely persist after 1 year. However, the patients may be protected lifelong from reinfection if they live in an endemic area of disease where they have persistent booster stimulations by typhoid bacilli from the environment.

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