ELISA for the Measurement of Intestinal Antibody to *Salmonella typhi* Protein Antigen*

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SUMMARY **An enzyme-linked immunosorbent assay for the determination of intestinal IgA specific to a protein antigen of *S. typhi* is described. The optimal concentration of the antigen was 5 μg/ml of protein. The optimal incubation conditions for antibody and conjugate were at 4°C and 30°C overnight, respectively. The assay was used successfully for the determination of specific intestinal IgA in 3 typhoid patients and 18 normal controls. The antibodies detected were < 70 a.a.u./mg total IgA in 94 per cent (17/18) of the normal controls and > 200 a.a.u./mg total IgA in the three typhoid patients. However, a high level of antibody (701.75 a.a.u./mg total IgA) was also detected in one normal individual.**

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Studies of immune responses to *S. typhi* infection have been carried out mostly on systemic immunity.[1-9] However, it has been demonstrated that serum antibodies are not relevant to protection against typhoid fever[10] and the role of cell-mediated immunity in protection has not been clearly established. On the other hand, very few studies have been carried out on local intestinal immunity in typhoid patients,[11] although it could possibly be an important contributory factor in protective immunity against typhoid fever. Radioimmunoassay (RIA) has been used in a previous study of local intestinal immunity in typhoid patients,[11] but it has the disadvantages of being biologically hazardous and using unstable isotopic reagents. We therefore developed an enzyme-linked immunosorbent assay (ELISA) as an alternative method to study the intestinal immune response to *S. typhi* infection.

MATERIALS AND METHODS

Subjects

Eighteen normal healthy individuals with negative Widal tests, one volunteer receiving a single dose of oral live-attenuated typhoid vaccine "Vivotif Berna", kindly supplied by Professor R. Germanier, Swiss serum and Vaccine Institute Berne) and 4 typhoid patients with *S. typhi* positive haemocultures were included in the study.

Intestinal lavage specimens

Single specimens were obtained from each subject except the vaccinated volunteer. For typhoid patients these were obtained two months after the onset of illness. For the volunteer, one specimen was obtained before and another three months after vaccination.

The method used to collect specimens was originally a procedure for cleansing the intestine prior to contrast radiography[12] which was modified by Sack and co-workers.[13] In brief, the subjects were asked to drink a large volume (approximately 3 litres) of an isotonic balanced salt solution which contained no nutrients. This resulted in a brief attack of diarrhoea. Specimens of approximately 500 ml watery stool each were collected over a period of two hours: Each specimen was inactivated immediately in a water bath at 56°C for 15 minutes, then filtered through a gauze and chilled in an ice bath.

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The filtrates were then centrifuged at 10,000xG for 10 minutes, filtered through a 0.45 μ millipore filter and concentrated approximately 25 fold by ultrafiltration through dialysis tubing under vacuum. The concentrated specimens were divided into 0.5 ml alliquots and stored at -70°C until used.

Determination of total intestinal IgA levels

The levels of total IgA in intestinal lavage specimens were determined by an ELISA, using rabbit anti-human IgA covalently bonded to polyacrylamide beads ("Immunobeads", Bio-Rad Laboratories, Glattburg, Switzerland), according to the method previously described by Sack and co-workers,[14] with slight modification. The conjugate used was swine anti-human IgA (heavy chain specific) immunoglobulins labelled with alkaline phosphatase (Orion Diagnostica, Helsinki, Finland) and the substrate was p-nitrophenylphosphate (Sigma Chemical Company, St. Louis, MO, U.S.A.). The standard used was pooled milk from healthy Thai women, containing 0.75 mg of IgA per millilitre.

Antigen

Protein antigen was prepared from *S. typhi* strain 0-901, which had been cultured on brain heart infusion agar at 37°C overnight, according to the method previously described by Barber and co-workers.[15] Briefly, washed and acetone-dried cells were extracted by veronal buffer pH 8.4 and the protein antigen (Barber's protein, BP) was purified by precipitation with trichloracetic acid.

ELISA for the determination of intestinal IgA specific to Barber's protein antigen

The antigen was diluted with 0.05 M carbonate buffer, pH 9.8, containing 0.1% sodium azide, to a protein concentration of 5 μg/ml. One hundred microlitres of the antigen was added to each well of Microelisa Immulon plates (Dynatech Produkta AG, Kloten, Switzerland) and the plates were then incubated at 37°C for 3 hours. After the antigen solution was removed, the plates were washed three times with 0.01 M phosphate buffered saline, pH 7.1, containing 0.15% Tween 20 (PBST) and tapped dry. One hundred microlitres samples of serially 5 fold dilutioned intestinal lavage in PBST were added to each well. Then the plates were incubated at 4°C overnight. Each dilution of a specimen was assayed in duplicate. After three washes with PBST, 100 μl of the conjugate, which was swine anti-human IgA (heavy chain specific) immunoglobulins labelled with alkaline phosphatase (Orion Diagnostica), was added. The plates were incubated at 30°C overnight. Excess conjugate was washed out three times with PBST. One hundred microlitres of p-nitrophenylphosphate (Sigma Chemical Company) dissolved in 0.05 M carbonate buffer, pH 9.8, containing 0.005 M $MgCl_2 .6H_2O$ to a concentration of 1 mg/ml, was then added to each well as the substrate. The enzyme-substrate reaction was allowed to take place at 30°C for 45 minutes before being terminated by the addition of 25 μl of 3 M NaOH. The absorbance of the colour developed was read spectrophotometrically at 405 nm with a Titertek Multiskan® (Flow Laboratories, GmbH, Bonn, West Germany).

In addition to a reference intestinal lavage specimen, a direct conjugate control (DCC) and a substrate blank (SB) were included in each plate. One hundred microlitres of PBST was substituted for intestinal lavage in the DCC and for both intestinal lavage and conjugate in the SB. The wells for the DCC provided a control for the extent of nonspecific conjugate binding and the wells for the SB provided a control for spontaneous hydrolysis of the substrate.

To calculate the results, the mean absorbance of each dilution minus the mean absorbance of the DCC, performed in the same plate, was used.

For the quantitation of specific IgA, a curve was constructed as a reference intestinal lavage specimen by plotting absorbance units against corresponding dilutions. The end point of the reaction tested was read at a point of 0.4 absorbance unit above the base line and the corresponding dilution which gave this end point reading was determined from the curve. For unknown specimens, curves were also constructed and end point dilutions were determined in the same manner. Since there were no recognised units for anti-BP antibodies, the reference lavage specimen was arbitrarily assigned 100 units. Thus, the titres for unknown specimens could be quantitated in terms of arbitrary antibody units (a.a.u.) relative to the reference specimen.

RESULTS

Standardisation of ELISA

Lavage specimens obtained from the volunteer before and after receiving oral live-attenuated typhoid vaccine were used for the standardisation assay.

The optimal BP antigen concentration for coating wells was determined by doing checkerboard titration with varying concentrations of BP antigen, ranging from 0.1 to 50 μg/ml of protein, and 5 fold serial dilutions of both positive and control specimens, ranging from 1:5 to 1:15,625. The maximum uptake of antibody was obtained with a BP antigen concentration of 5 μg/ml, as illustrated in Figure 1. This concentration of BP antigen was then selected as the optimal antigen-coating concentration.

The effect of temperature and duration of antibody incubation on antibody binding to the coating antigen was investigated at 4°C, 30°C and 37°C for 1, 3, 5 and 24 hours. The highest absorbance reading was obtained when the incubation was at 4°C for 24 hours, as shown in

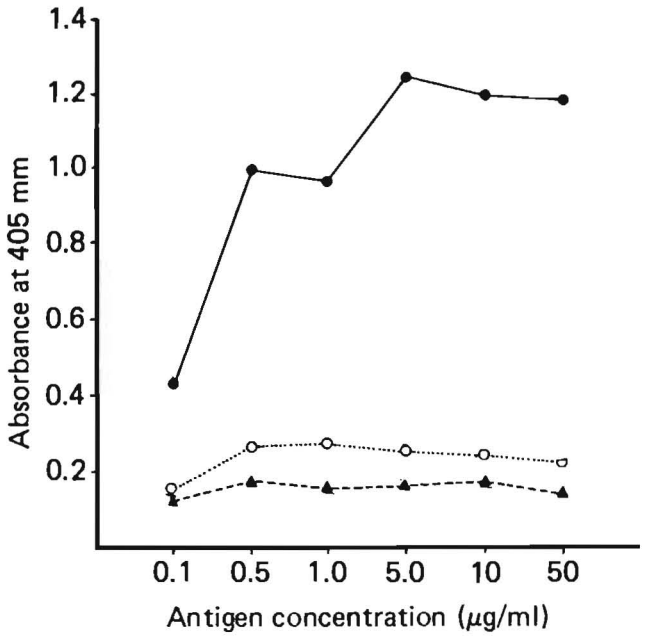

Fig. 1 Optimal antigen concentration for the maximum uptake of specific antibody. Plates were coated with 100 μl of antigen, concentration ranging from 0.1 to 50 μg/ml. Antibody and conjugate dilutions were 1:5 and 1:100 and incubation periods were 5 hours and 18 hours, at 30°C, respectively.

● = Human intestinal lavage specimen obtained 3 months after oral typhoid vaccination.

○ = Normal human intestinal lavage specimen.

▲ = Direct conjugate control.

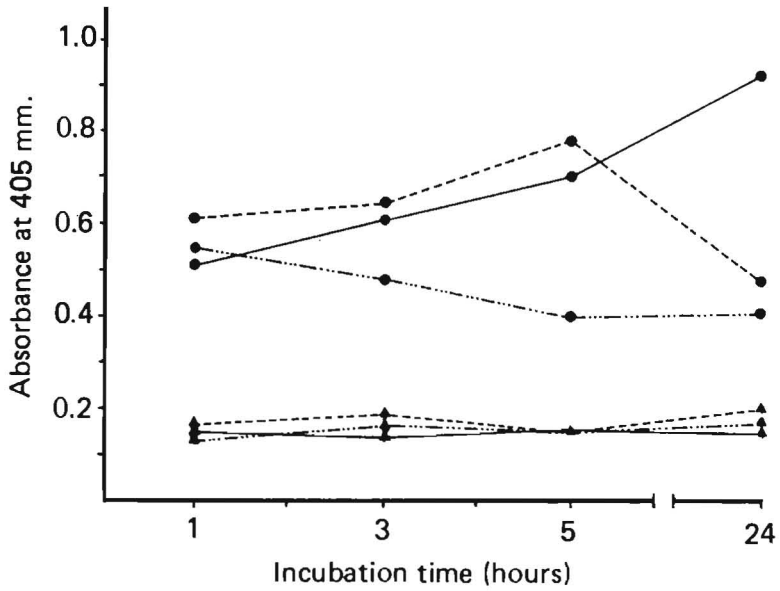

Fig. 2 Optimal antibody binding. Plates were coated with 100 μl of antigen, concentration 5 μg/ml. The antibody dilution was 1 in 5 and the incubation period and temperatures were 1, 3, 5 and 24 hours, at 4°C (———), 30°C (·····) and 37°C (—·—·—). Conjugate dilution and incubation conditions were 1 in 100 and 20 hours at 4°C.

● = Human intestinal lavage specimen obtained 3 months after oral typhoid vaccination.

▲ = Direct conjugate control.

Figure 2. For practical purposes, overnight incubation with antibody at 4°C was selected. The optimal temperature and duration for conjugate incubation were determined in a similar manner. The results are illustrated in Figure 3. The maximum uptake of conjugate was obtained by 24-hour incubation at 30°C.

Using the optimal conditions outlined above, a reference intestinal lavage specimen obtained from one of the four typhoid patients two months after onset of illness was assayed for IgA specific to BP antigen. A typical curve obtained from the assay of five fold serial dilutions of this reference specimen is shown in Figure 4. The highest dilution of this specimen which gave measurable colour was 1:125. The significant extinction was then selected to be the midpoint between the absorbance unit registered with this dilution 1: 125 and that of the more concentrated specimen next to it (1:25). The difference between the significant extinction and the mean absorbance at the base line of the same curve was determined from 10 experiments. The mean of the differences was then calculated and found to be 0.4. This value was used to indicate the end point of each specimen reaction, i.e., the end point was determined at a point 0.4 absorbance units above the base line.

Reproducibility

The reproducibility of the technique was examined by testing the reference specimen 30 times. The mean end-point value had a standard deviation of 0.01 absorbance units. In addition the titre which gave the end point antibody reaction was reproducible within a 2.2 fold range.

Assay of test specimens

The ELISA developed was used for the determination of intestinal IgA specific to BP antigen in lavage specimens obtained from 18 normal individuals and from three typhoid

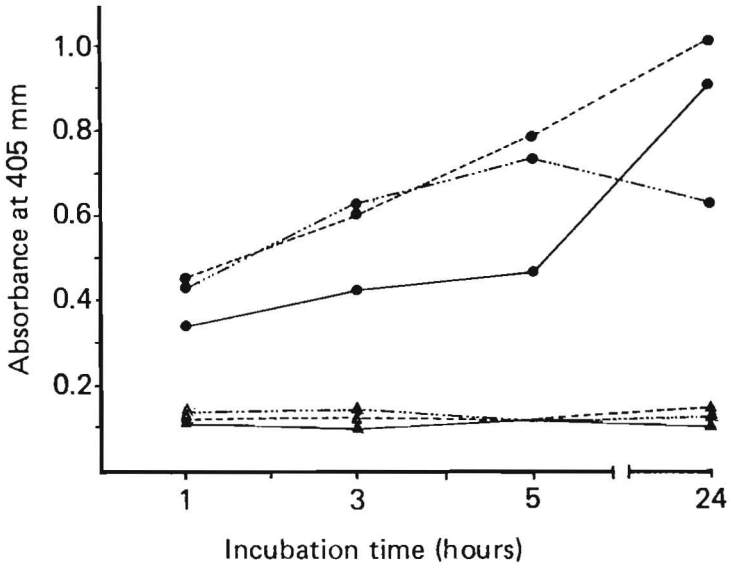

Fig. 3 Optimal conjugate binding. Plates were coated with 100 μl of antigen, concentration 5 μg/ml. The antibody dilution and incubation conditions were 1 in 5 and 20 hours at 4°C, respectively. Conjugate dilution was 1 in 100 and the incubation periods and temperatures were 1, 3, 5 and 24 hours at 4°C (———), 30°C (- - - -) and 37°C (–·—·–).

● = Human intestinal lavage specimen obtained 3 months after oral typhoid vaccination.

▲ = Direct conjugate control.

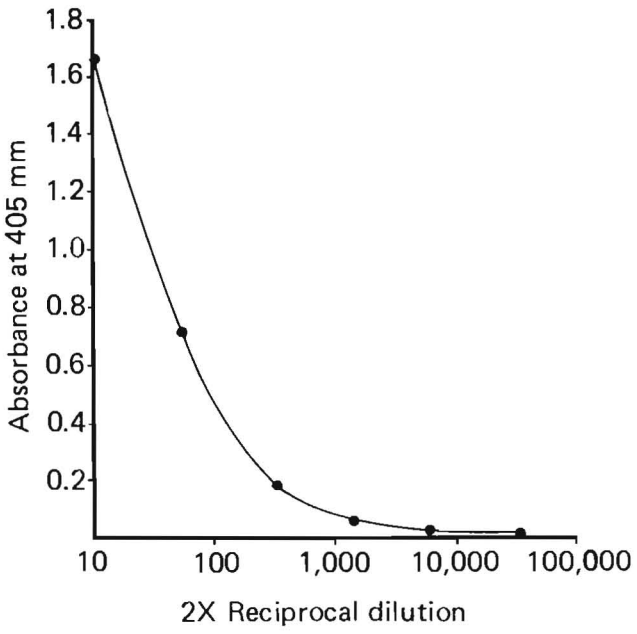

Fig. 4 A typical curve of the reference intestinal lavage specimen. Plates were coated with 100 μl of antigen, concentration 5 μg/ml. Antibody dilutions ranged from 1 in 5 to 1 in 625 and incubation conditions were overnight at 4°C. Conjugate dilution and incubation conditions were 1 in 100 and overnight at 30°C, respectively. Substrate incubation was 45 minutes at 30°C.

patients. The reference lavage specimen was included in each micro ELISA plate to permit adjustment of results obtained in experiments performed on different occasions or even in different plates. The reference specimen was arbitrarily assigned 100 units, as there were no recognised units for anti-BP antibody. By relating the end-point dilutions of test samples to that of the reference sample tested in the same plate, the titres of the test samples could be determined and expressed as arbitrary antibody units (a.a.u.).

The total IgA in lavage specimens may vary significantly, not only between different persons, but also between different specimens from one individual, depending on dilution by water. Since the amount of total IgA present influenced the titre of specific IgA determined in each specimen, it was necessary to adjust antibody titres relative to the total IgA giving final titres as a.a.u. of antibody per mg of total IgA. This corrected for differences in initial dilution factors. It was then possible to compare the quantities of specific IgA present among the various samples. The results are shown in Table 1 and Figure 5. It can be seen that lavage specimens from 17 out of 18 normal control subjects contained anti-BP IgA less than 70 a.a.u./mg total IgA. One individual had 701.75 a.a.u./mg total IgA. For the typhoid patients 2 of 3 had intestinal anti-BP IgA > 700 a.a.u./mg total IgA while the other had an antibody level of 295.49 a.a.u./mg total IgA.

DISCUSSION

Although typhoid fever is well known as a disease with primary lesions in the gastrointestinal tract, the role of local intestinal immunity in protection against the disease has not been well elucidated. A study of local intestinal immunity in typhoid patients has been carried out recently with RIA,[11] but this technique is biologically hazardous

Table 1 Anti-BP-IgA levels and total amount of IgA in 1 ml of intestinal lavage specimens obtained from 18 normal subjects and from three typhoid patients two months after the onset of illness.

Normal controls			Typhoid patients		
anti-BP-IgA (a.a.u.)	total IgA (mg)	anti-BP IgA/ mg total IgA	anti-BP-IgA (a.a.u.)	total IgA (mg)	anti-BP IgA/ mg total IgA
6.11	0.825	7.40	69.44	0.235	295.49
<6.06	0.450	<13.47	488.89	0.695	703.44
<6.25	0.450	<13.89	238.89	0.285	838.21
37.75	2.385	15.83			
12.31	0.455	27.05			
14.21	0.375	37.89			
<9.50	0.153	<62.09			
<7.69	0.320	<24.03			
15.38	0.225	68.35			
<11.10	0.208	<53.37			
12.86	0.415	30.98			
<8.70	0.275	<31.64			
15.38	0.840	18.31			
16.88	0.849	19.88			
200.00	0.285	701.75			
36.00	0.580	62.07			
31.58	0.790	39.97			
16.92	0.621	27.25			

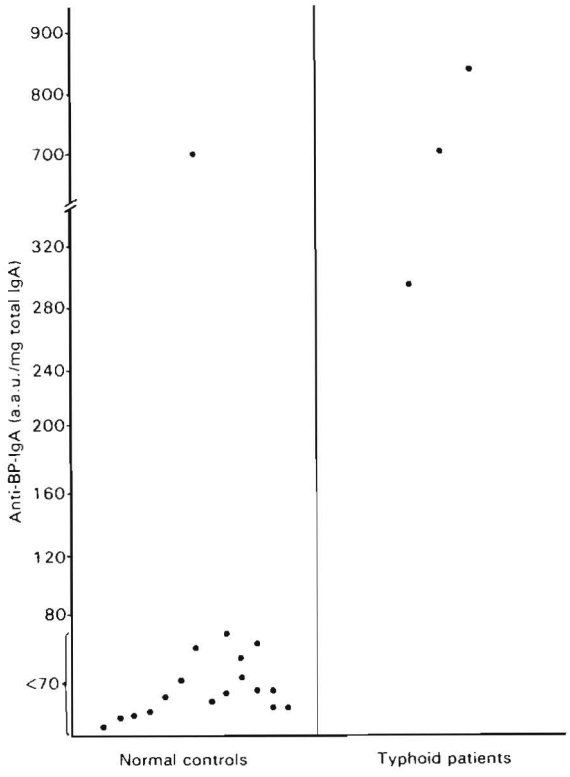

Fig. 5 Anti-BP-IgA levels in intestinal lavage specimens obtained from normal subjects and from typhoid patients two months after the onset of illness.

and uses an unstable isotopic reagent. We, therefore, developed an indirect ELISA as an alternative method for further study of the role of the local intestinal immunity in typhoid fever and the technique has proven to be both sensitive and reproducible.

With this technique, we found that approximately 94 per cent (17/18) of normal control subjects had intestinal anti-BP IgA lower than 70 a.a.u./mg total IgA, while all three typhoid patients developed intestinal antibody responses with levels greater than 200 a.a.u./mg total IgA. However, one normal control subject had an antibody level of 701.75 a.a.u./mg total IgA, even though he had a negative Widal test. This individual could possibly be previously infected by *S. typhi* but with serum antibodies decreased to a non-detectable level and intestinal antibodies still persistent. If this is the case, it indicates that intestinal antibody developed after typhoid infection may persist for a long time and may be important in protective immunity against typhoid fever.

Since very few typhoid patients were available for lavage specimen collection for this study we cannot draw any definite conclusions concerning the role of local intestinal immunity in typhoid fever. However, because of its sensitivity and reproducibility, the ELISA technique reported here is potentially valuable for further investigations on local intestinal immunity against typhoid fever.

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