Antibodies to Porin Antigens of *Salmonella typhi* Induced during Typhoid Infection in Humans

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Immunoglobulin G (IgG)- and IgM-specific antibody titers against *Salmonella typhi* Ty2 porins have been measured in 30 paired typhoid sera by enzyme-linked immunosorbent assay. These studies have found that IgG serum titers of acute and convalescent sera were 625 and 5,000 times higher, respectively than the control serum titers. The same typhoid sera were titrated with *S. typhi* Ty2 flagellin and *S. typhi* lipopolysaccharide. The titers against these antigens were considerably lower than those against the porins. The highest IgM-specific titer has also been found against porins in convalescent-phase sera. However, the largest increase in IgM-specific titer compared with the control group titer was obtained against flagellin during the acute phase of typhoid. The lowest increases in antibody titer were obtained with the IgM-specific anti-lipopolysaccharide in both types of sera. This may be because many normal individuals in endemic areas already have IgM titers against lipopolysaccharide. This study has provided good evidence that porins are excellent antigens and that IgG-specific antiporin titers may be of diagnostic value in typhoid infections in endemic areas.

Studies of the antibody response to *Salmonella typhi* during a typhoid infection in humans have frequently been restricted to the detection of antibodies induced by a small number of bacterial antigens. Thus the well known Vi antigen (capsular polysaccharide), H antigen (flagellar component), and O antigen (lipopolysaccharide [LPS]) have been responsible for agglutinations of bacteria, erythrocytes, and antigen-coated inert particles (8, 19). These reactions detected primarily immunoglobulin M (IgM) antibodies. Nevertheless, the three major immunoglobulin types against Vi, H, and O antigens have been demonstrated by using DEAE-fractionated (6) and 2-mercaptoethanol-reduced (14) sera.

In recent years more specific and sensitive techniques than agglutination, such as radioimmunoassay and enzyme-linked immunosorbent assay (ELISA), have been used for detecting and quantitating the antibody levels primarily to *Salmonella* sp. O antigens (1, 5, 22, 28). Although attempts have been made to characterize the humoral response to protein antigens other than the flagellar component, these have been performed either with poorly defined antigens or with mixed-antigen preparations (1, 26, 28). The porins are examples of such antigens that have not been systematically studied. A number of recent reports have shown that the outer membrane proteins, porins among them, play a role in pathogenesis and are important antigens against which the host immune response is directed (3, 7, 9–11, 15, 17, 18, 27). In this work we were interested in evaluating the humoral immune response elicited in typhoid fever patients against porins. We have measured IgG- as well as IgM-specific antiporin antibodies by ELISA. We have also compared the porin-induced antibody levels with those elicited by flagellin and by LPS in the same serum preparations.

MATERIALS AND METHODS

Bacterial strains. *S. typhi* Ty2 was a kind gift from the Instituto de Salud Pública de Chile.

Flagellin preparation. Flagellin was obtained from *S. typhi* Ty2 according to the procedure of Nossal and Ada (24), who used mild agitation of the bacteria and acidification of the flagellar suspension with 0.1 N HCl to yield flagellin.

Isolation of *S. typhi* Ty2 porins. Bacteria were grown in Carlquist ninhydrin base medium (Fisher Scientific Co.) with 1.5% agar. A yield of approximately 25 g (wet weight) of cells was harvested. Outer membranes were obtained by the procedure of Moore et al. (21). Porins OmpF and OmpC were isolated by the method of Calderón et al. (4) and then were suspended in 200 mM Tris hydrochloride buffer (pH 7.5) and alkylated with 10 mM pyridoxal phosphate at 37°C for 2 h. This procedure yields solubilized porin preparations. Thereafter the porins were reduced with 0.5 mg of sodium borohydride (13) per ml and extensively dialyzed against 20 mM $NaHCO_3$, pH 7.8.

Chemical assays. Protein concentrations were measured by a modification of the method of Lowry et al. (20). LPS were determined by the thiobarbituric acid method modified by Osborn (25) and by the *Limulus* amebocyte lysate assay procedure (30). LPS from Sigma Chemical Co. was used as a standard in these assays. The sensitivity of the *Limulus* amebocyte lysate assay was 1 ng of LPS per ml.

ELISA. The ELISA has been performed by standard methods (29), using micro-ELISA plates. The wells of disposable flexible polyvinyl microtitration plates (Dynatech Laboratories, Inc.) were coated with 0.05 ml of each antigen and incubated overnight at room temperature. The plates were washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (Sigma), and the wells were filled to the top with PBS containing 1% bovine serum albumin and 0.02% sodium azide and then incubated at room temperature for 1 h. The plates were washed again with PBS-Tween 20 solution and loaded with 0.05-ml samples of sera serially diluted in PBS-bovine serum albumin, using 10-fold dilution steps. The plates were incubated at 37°C for 1 h and then washed. This was followed by incubation with heavy chain-specific antibody to human IgM or IgG conjugated with alkaline phosphatase (Sigma) for 3 h at 37°C.

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Then the fluid was discarded, and the wells were again washed three times with PBS-Tween 20. A 0.05-ml portion of substrate consisting of *p*-nitrophenylphosphate (Sigma) adjusted to a concentration of 1 mg/ml in 0.05 M carbonate buffer (pH 9.6) containing 0.001 M $MgCl_2$ was added to each well. The reaction was stopped after incubation for 30 min at room temperature by the addition of 0.05 ml of 3 N NaOH. The titer was considered to be the 10-fold dilution of sample giving an A_{400} of 0.2. Individual antigens were tested according to Keren (12) for optimal coating concentration, i.e., the antigen concentration giving the highest antibody value. These values were 4 μg/ml in PBS for porin preparations, 10 μg/ml in PBS for flagellin preparations, and 10 μg/ml in 0.05 M carbonate buffer (pH 9.6) for LPS. *S. typhi* LPS was purchased from Sigma.

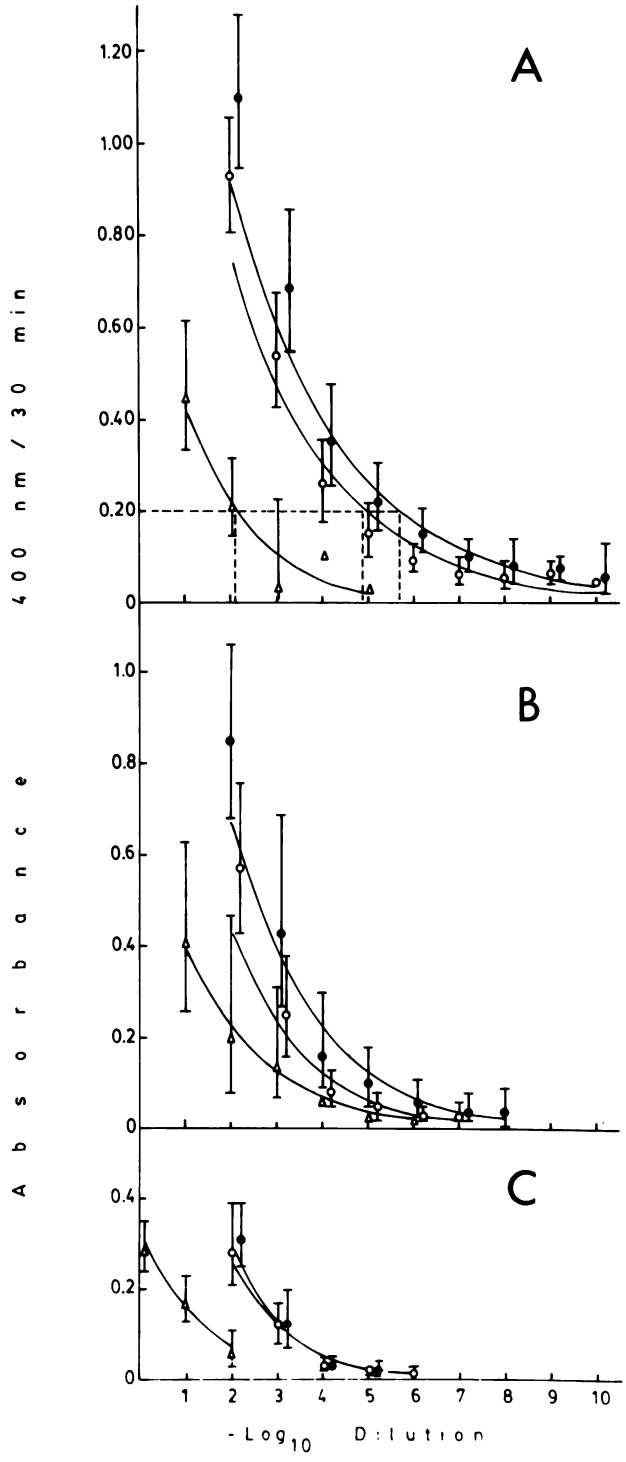

FIG. 1. IgG titration of human sera in ELISA against *S. typhi* antigens. The wells were coated with 0.05 ml of sample containing: (A) 4.0 μg of OmpF and OmpC porins per ml, (B) 10 μg of flagellin per ml, and (C) 10 μg of LPS per ml. The continuous tracings represent the best theorethical exponential fitting curves obtained from each set of experimental data. Exponential curves of the kind $y = a \cdot e^{bx}$ were found to describe the titration process when the geometric means of the absorbance at every dilution were plotted. In every curve, the validity of the regression coefficient r was assessed, and $P < 0.001$ was found in all of them. The absorbance was measured in duplicate. Symbols: ○, acute-phase sera; ●, convalescent-phase sera; △, control sera. The titer is indicated as the 10-fold dilution to a final absorbance of 0.2. The vertical bars show the 95% confidence limits.

Serum specimens. Serum samples were obtained from 45 individuals (20 to 40 years old). Thirty samples came from individuals with typhoid fever, confirmed by positive blood culture, who were hospitalized at the Hospital de Enfermedades Infecciosas, Santiago, Chile; the other 15 serum samples (control group) came from volunteers who had never been infected (clinically confirmed) with any type of *Salmonella* sp. and who came from the same endemic area as the former group. The so-called acute-phase sera were collected on day 1 of hospitalization of individuals of the typhoid group, and 15 days later the convalescent-phase sera were obtained.

Statistical analysis. The data were analyzed as the $\log_{10}$ of the titers, using standard statistical procedures with a BASIC program in a CASIO FX-750-P personal microcomputer. Geometric mean values of individual titers were used for assessment of the data. For statistical significance studies, antiporin, antiflagellin and anti-LPS IgG titers were compared by the Kruskal-Wallis test (chi square approximation). The Wilcoxon two-sample test (normal approximation) was used to compare these titers in pairs.

RESULTS

The antibody levels to porin were measured by ELISA, using paired sera from the typhoid patients, sera from the control group, and copurified *S. typhi* Ty 2 porins OmpF and OmpC.

Figure 1A shows the titration process of antiporin IgG in control, acute, and convalescent sera. Geometric means of the absorbance at every dilution were plotted. As predicted by theory, there was a highly significant correlation coefficient to an exponential function ($P < 0.001$).

In this manner, geometric mean antiporin IgG titers of 1.5×10^{-5} and 2.1×10^{-6} were found for acute and convalescent sera, respectively. These sera were found to be 625 and 5,000 times higher, when compared with the geometric mean antiporin IgG titer of 9.3×10^{-3} measured in control group sera (Table 1). Similar titers of antibody against outer membrane proteins of *Pseudomonas aeruginosa* have been measured by ELISA in sera of patients with cystic fibrosis (10).

Antibody levels to H and O antigens were quantitated in the same groups of sera (Fig. 1B and C, respectively). By using *S. typhi* Ty2 flagellin, geometric mean titers of 6.5

TABLE 1. ELISA titration of human IgG antisera with *Salmonella* antigens

Source of sera	Geometric mean titer[a]		
	Porin antigens	H antigen	O antigen
Control	9.3×10^{-3} (1.5×10^{-2}–6.3×10^{-3})	7.3×10^{-3} (1.2×10^{-2}–4.9×10^{-3})	2.6×10^{-1} (4.3×10^{-1}–1.8×10^{-1})
Acute phase	1.5×10^{-5} (1.7×10^{-5}–1.3×10^{-5})	6.5×10^{-4} (7.8×10^{-4}–5.5×10^{-4})	5.2×10^{-3} (6.6×10^{-3}–4.2×10^{-3})
Convalescent phase	2.1×10^{-6} (2.5×10^{-6}–1.8×10^{-6})	1.4×10^{-4} (1.7×10^{-4}–1.2×10^{-4})	3.8×10^{-3} (5.1×10^{-3}–2.9×10^{-3})

[a] Values shown in parentheses represent the confidence intervals for the geometric mean titer, with a 95% confidence level.

$\times 10^{-4}$ for the acute-phase sera and 1.4×10^{-4} for the convalescent-phase sera were found. The geometric mean H-antigen serum titer of the control group was 7.3×10^{-3}; thus, during illness the H-antigen titer increased only 11 and 54 times, respectively.

S. typhi LPS has also been used in the ELISA measurements of typhoidal sera, and a geometric mean titer of IgG-specific antibody of 2.6×10^{-1} was detected in the control group (Fig. 1C). This reflects a low level of IgG antibody induction by LPS in individuals from an endemic area such as Chile. However, during typhoid fever, geometric mean ELISA titers of 5.2×10^{-3} for acute-phase sera and 3.8×10^{-3} for convalescent-phase sera were found (Table 1).

Similar results have been obtained for acute typhoid sera by Carlsson et al. (5), who found, by ELISA, mean titers against LPS 10 to 20 times higher in patients than in healthy blood donors.

There was a statistically significant difference between the geometric mean titration curves of antiporin, antiflagellin, and anti-LPS IgG sera of patients in both the acute ($P < 0.0001$) and the convalescent ($P < 0.0001$) phases, compared by the Kruskal-Wallis test (chi square approximation). The geometric mean titer of antiporin was compared with those of antiflagellin and anti-LPS, all from acute-phase sera, by the Wilcoxon two-sample test, and it resulted in $P < 0.0006$ and $P < 0.0001$, respectively. When the same comparison was done with convalescent-phase sera, values of $P < 0.004$ and $P < 0.001$ were obtained, respectively.

Determinations analogous to those described for IgG class antibodies were performed on the same group of paired sera to determine the respective IgM titers for three *Salmonella* sp. antigens (Table 2). Thus, it was found that IgM geometric mean titers against porins increased 6.4 times during the acute phase and 36 times during the convalescent phase. The antiflagellin IgM titers were found to be lower in convalescence than in the acute phase of typhoid, while the later geometric mean titer was 88 times higher in patients than in the controls. Finally, the IgM titers against LPS are shown to have undergone a slight increase (two- to threefold) during illness compared with the normal group of volunteers.

DISCUSSION

Remarkably high antiserum titers against porins have been found in typhoid fever patients. The use of solubilized porin preparations instead of sodium dodecyl sulfate-free porin aggregates were critical for the success of antiserum titrations in ELISA (29). The use of sodium dodecyl sulfate-free porin aggregates did not allow us to develop an adequate standardization of the system. At that state of aggregation, the optimal amount of porins available as antigens for coating of the wells could not be ensured. This was reflected in highly variable ELISA absorbancy measurements.

It was also found that IgG antiporin titers during the convalescent phase were several times higher than those in the acute-phase sera. Both titers in turn were found to be many times higher than the control group IgG titers. All of these facts suggest that the detection of IgG antibodies against porins may be of diagnostic value for typhoid fever.

It is significant that previous ELISA titrations that have used *Salmonella* protein antigens had failed to detect any difference in the level of antibodies induced during the acute and convalescent phases (26).

With LPS antigen it has been shown that the corresponding IgG antibody titers had increased several times during the illness, but this has not been the case for the IgM-specific LPS antibody titers. The latter finding resulted from taking into consideration the control serum IgM anti-LPS titers. Therefore, it can be concluded that normal individuals from an endemic area have IgM antibody levels already too high to expect large increases during illness. Similar conclusions have been reached in studies of the Widal test in areas endemic for typhoid fever (16).

As expected for a protein antigen, the IgM titer against flagellin during the convalescent phase was found to be significantly lower than the titer found in sera collected at the beginning of the typhoid infection. However, IgM antiporin antibodies have not followed the same trend. It appears that porins are excellent antigens interacting efficiently with the host immune system. Among the known properties of porins that enhance their antigenicity is the resistance to proteolytic enzymes, which assures lasting stability in the circulation of the host. Also, the affinity of porins for artificial lipid membranes (2), for liposomes (23), and for the human erythrocyte membranes (4) favor the possibility that the porins may also interact with macrophages in ways that strongly stimulate the immune response. In support of this notion is the report that the macrophage-mediated tumor cell killing is enhanced by porins by way of a putative interaction

TABLE 2. Elisa titration of human IgM antisera with *Salmonella* antigens

Source of sera	Geometric mean titer[a]		
	Porin antigens	H antigen	O antigen
Control	1.4×10^{-2} (2.3×10^{-2}–9.0×10^{-1})	2.9×10^{-2} (4.9×10^{-2}–1.9×10^{-2})	7.0×10^{-3} (1.7×10^{-2}–3.8×10^{-3})
Acute phase	2.2×10^{-3} (3.1×10^{-3}–1.6×10^{-3})	3.3×10^{-4} (4.7×10^{-4}–2.4×10^{-4})	3.6×10^{-3} (4.9×10^{-3}–2.7×10^{-3})
Convalescent phase	3.7×10^{-4} (5.5×10^{-4}–2.7×10^{-4})	1.7×10^{-3} (2.5×10^{-3}–1.2×10^{-3})	2.4×10^{-3} (3.4×10^{-3}–1.8×10^{-3})

[a] Values shown in parentheses represent the confidence intervals for the geometric mean titer, with a 95% confidence level.

with macrophage (31). Such reasoning would suggest the attempt to use porins as potential immunogens or vaccines.

ACKNOWLEDGMENTS

We thank Felix Wassermann, Felipe Cabello, and Arnaldo Foradori for the critical reading of this manuscript. We are extremely grateful to Adriana Contreras (QF) Jefe de la Sección DPT from the Instituto de Salud Pública de Chile for technical cooperation, to Mario Alvarado for statistical assistance, and to Benito Gómez for discussions concerning this manuscript.

This research was supported by grant 74/85 from the Dirección de Investigación, Pontificia Universidad Católica de Chile and by grant 1176/85 from Conicyt.

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