Clin. exp. Immunol. (1981) **46**, 508–514.

Antibody response to the lipopolysaccharide and protein antigens of *Salmonella typhi* during typhoid infection

I. MEASUREMENT OF SERUM ANTIBODIES BY RADIOIMMUNOASSAY

R. S. W. TSANG,* P. Y. CHAU,* S. K. LAM,† J. T. LA BROOY‡ & D. ROWLEY§
*Departments of *Microbiology and †Medicine, University of Hong Kong, Hong Kong; and Departments of ‡Medicine and §Microbiology & Immunology, University of Adelaide, South Australia*

(Accepted for publication 12 June 1981)

SUMMARY

Serum antibody responses to the lipopolysaccharide and protein antigens of *S. typhi* in typhoid patients were studied using a solid-phase radioimmunoassay technique. Sera from 24 adult typhoid patients and 20 non-typhoid adult controls were compared. As a group, sera from typhoid patients showed increased IgA, IgG and IgM immunoglobulin levels and gave significantly higher anti-LPS and anti-protein antibody titres in all three major immunoglobulin classes than did non-typhoid controls. Levels of antibodies against LPS or protein in sera of typhoid patients were highly variable with a skew distribution. A good correlation was found between antibody titres to the LPS antigen and those to a protein antigen. No correlation, however, was found between the anti-LPS antibody titres measured by radioimmunoassay and the anti-O antibody titres measured by the Widal agglutination test. Titration of anti-LPS or anti-protein antibodies by radioimmunoassay was found to be more sensitive and specific than Widal test for the serological diagnosis of typhoid fever. The advantages of measuring antibody response by radioimmunoassay over conventional Widal test are discussed.

INTRODUCTION

Our knowledge of the humoral immune response to antigens of *Salmonella typhi* during typhoid infection has been limited to data obtained by bacterial agglutination or indirect agglutination using somatic antigens coated onto red blood cells or other inert particles. Using these tests, LoSpalluto *et al.* (1962) and Fink *et al.* (1962) reported that anti-*S. typhi* O antibody activity was exclusively in the IgM immunoglobulin class, Turner & Rowe (1964) found such antibodies in both the IgM and IgA classes, while Rossen, Wolff & Butler (1967), Chernokhvostova *et al.* (1969) and Kumar *et al.* (1974) detected anti-O antibody activity in all three major immunoglobulin classes (IgA, IgG and IgM). Similar studies of host antibody response to other Gram-negative bacterial O antigens have also produced divergent results (Weidanz, Jackson & Landy, 1964; Fukazawa *et al.*, 1970; Gannon *et al.*, 1980). An assay of antibodies based on their primary binding to the corresponding antigens, such as radioimmunoassay, should help to clarify the issue.

The lipopolysaccharide (LPS) antigens of Salmonella used to be regarded as the major antigenic determinants of the somatic O antigen (Jawetz, Melnick & Adelberg, 1978). It has now been shown that Salmonella organisms, like other Gram-negative bacteria, also contain protein antigens besides

Correspondence: Dr P. Y. Chau, Department of Microbiology, Pathology Building, Queen Mary Hospital Compound, Hong Kong.

0009-9104/81/1200-0508$02.00 © 1981 Blackwell Scientific Publications

the LPS antigen (Barber & Eylan, 1974, 1976). In animal studies, protective immunity elicited by such protein antigens has been demonstrated (Barber & Eylan, 1975; Plant, Glynn & Wilson, 1978; Kuusi *et al.*, 1979). Despite these findings, studies to measure the immune response of typhoid patients to protein antigens of *S. typhi* are lacking. We therefore studied the antibody response during typhoid infection to the protein and LPS antigens of *S. typhi* by a solid-phase radioimmunoassay technique.

MATERIALS AND METHODS

Patients and serum specimens. Blood specimens were collected from 24 adult typhoid patients confirmed by positive blood culture and 20 normal adult controls (laboratory staff and non-typhoid patients) who had no previous history of typhoid fever and typhoid vaccination. Samples were taken once in controls and once or twice in patients. The first sample from each patient was collected as early as possible but usually after the first week of illness, and the second where available was collected 1–2 weeks later. Serum was separated and stored in aliquots at −20°C until use.

Preparation of antigens. A freshly isolated strain of *S. typhi* was grown on brain heart infusion agar in Roux bottles overnight. The growth was harvested in saline and poured into three volumes of acetone for inactivation. These bacteria were centrifuged down and further washed with acetone. The LPS antigen of *S. typhi* was extracted from acetone-dried cells by the hot phenol–water method of Westphal & Jann (1965), while the protein antigen (Barber's protein) was extracted from the acetone-dried cells by veronal buffer and purified by repeated precipitation with trichloroacetic acid (TCA) according to the method of Barber (1961).

Bacterial agglutination test. Anti-*S. typhi* O and H antibodies were measured by conventional tube method against stained antigen suspensions purchased from Wellcome Laboratories. Agglutination titre was defined as the reciprocal of the highest dilution of serum giving definite agglutination after overnight incubation at 37°C.

Determination of serum immunoglobulin levels. Serum IgA, IgG and IgM levels were determined by single radial immunodiffusion technique (Mancini *et al.*, 1964) using heavy chain-specific antisera to IgA, IgG and IgM (Behringwerke, West Germany). Human IgA, IgG and IgM reference sera (Hyland, USA) were used as standards.

Solid-phase radioimmunoassay (SP-RIA). Serum antibody content was determined by SP-RIA according to the method of Zollinger, Dalrymple & Artenstein (1976) with minor modifications. Briefly, polyvinyl microtitre plates (Cooke Engineering Co., USA) were coated with protein or LPS antigens at a concentration of 10 μg/50 μl for 1 hr in a moist chamber at 37°C. Unoccupied binding sites on the plastic were blocked by 1% bovine serum albumin (Sigma) in phosphate-buffered saline (PBS), pH 7·4. Wells were then washed twice with PBS. Serial dilutions of serum were added in 50 μl volume to appropriate antigen-coated wells as well as to antigen-free BSA-treated control plates. After incubation in a moist chamber at 4°C overnight, sera were removed and wells washed once with 1% BSA and three times with PBS. ^{125}I-labelled heavy chain-specific anti-human IgA, IgG, IgM and anti-human light chain antibodies, purified and labelled as described by La Brooy, Rowley & Shearman (1980), were then added to the appropriate wells (40 ng in 50 μl volume per well) and the plates were incubated in a moist chamber at 4°C overnight. After removal of the secondary antibodies, the wells were washed once with 1% BSA and four times with PBS, and cut out for counting of radioactivity in a Beckman G4000 gamma counter. The amount of secondary antibody bound was obtained by subtracting radioactivity bound to wells without antigen from those of antigen-coated wells. For the estimation of total anti-LPS or anti-protein antibodies, labelled anti-light chain antibody was used as secondary antibody whilst the antibodies in IgA, IgG and IgM classes were measured by labelled anti-alpha, anti-gamma and anti-mu antibodies. Antibody titre was arbitrarily defined as the reciprocal dilution of serum at which 2 ng of the ^{125}I-labelled anti-immunoglobulin antibody bound. Titres were also expressed in units per mg of immunoglobulin to compensate for the difference of immunoglobulin levels in sera of individual patients and controls.

RESULTS

Serum immunoglobulin levels in typhoid patients

In Fig. 1, the immunoglobulin levels of typhoid patients were compared with those of healthy controls. Typhoid patients showed a progressive increase in IgA, IgG and IgM immunoglobulin levels which extended for at least 8 weeks.

Antibody response to the proteins and LPS antigens of S. typhi *during typhoid infection*

As a group, typhoid patients had significantly elevated antibodies to both the protein and LPS

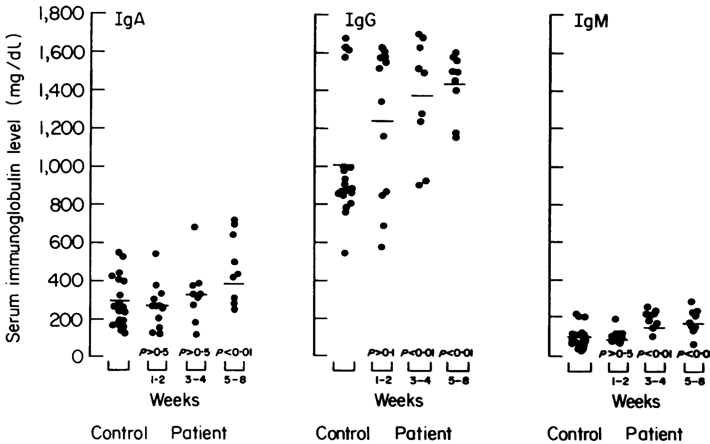

Fig. 1. IgA, IgG and IgM immunoglobulin levels in sera of typhoid patients and non-typhoid controls. (———) Mean immunoglobulin level; P values given were tested against non-typhoid controls.

antigens and these antibodies were found in all three major classes of immunoglobulins (IgA, IgG and IgM). However, the antibody levels in typhoid patients were found to be extremely variable with some patients, who had been ill for 1–6 weeks, having antibody titres comparable to those in the controls. There was thus a minor overlap between the antibody levels in sera of patients and those of controls (Figs 2 & 3).

By arbitrarily choosing an antibody titre in each class of immunoglobulin as the diagnostic level—e.g. 1,500 for the total antibodies, 75 for IgA, 750 for IgG and 300 for IgM-class antibodies—90–94% of typhoid patients were found to have anti-LPS antibody titres and 87–100% to have anti-protein antibody titres above the diagnostic level (Table 1). Among the normal controls, the most commonly occurring antibodies were IgM-class anti-LPS and IgG-class anti-protein antibodies. Using anti-human light chain to measure total antibodies, none of the controls yielded antibody titres above diagnostic level. Thus the best differentiation between typhoid patients and normal controls was found when anti-human light chain antibody was used to measure either the anti-LPS or the anti-protein antibody levels.

Correlation between the anti-LPS and anti-protein antibody levels in typhoid patients' sera

There was in general a good positive correlation between the level of the anti-protein antibodies with that of the anti-LPS antibodies in sera of typhoid patients. The correlation coefficient, (r) for the total antibodies to these two antigens as measured by anti-human light chain was 0·86. The r values for anti-LPS and anti-protein antibodies of the IgA, IgG and IgM classes were 0·64, 0·84 and 0·78 respectively. Despite this generally good correlation, a few patients exhibited discordance in their antibody response to these two types of antigens (marked with arrows in Fig. 4), particularly with the IgA-class antibodies: some patients were found to have high levels of anti-LPS antibodies but low levels of anti-protein antibodies while the reverse was true for a few other patients.

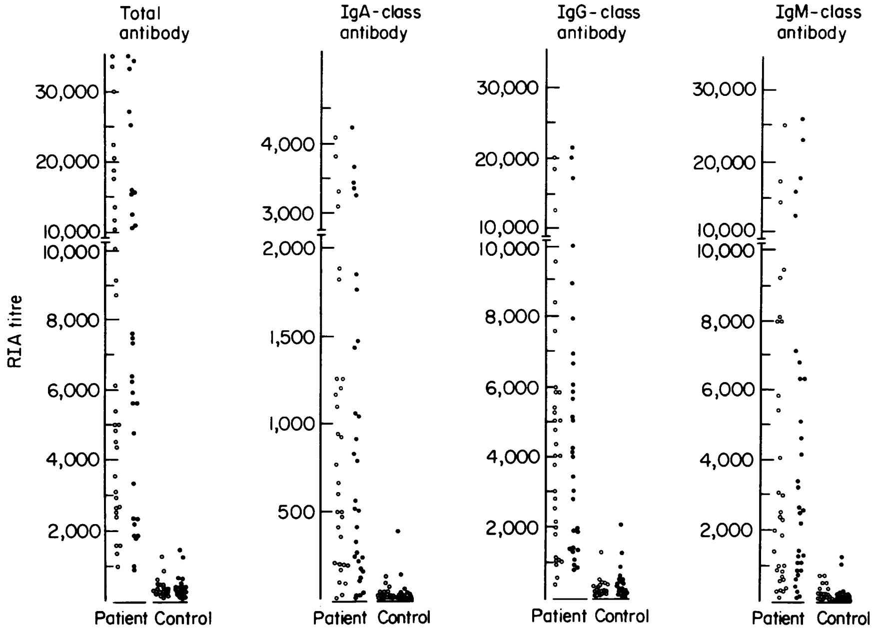

Fig. 2. RIA titres of antibodies to the proteins and LPS antigens of *S. typhi* in sera of typhoid patients and non-typhoid controls. (o) Anti-LPS antibodies, (•) anti-protein antibodies.

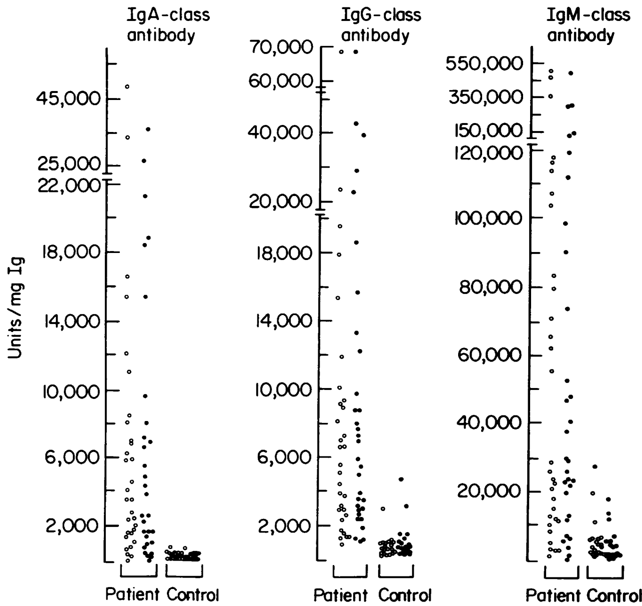

Fig. 3. RIA titres of antibodies to the proteins and LPS antigens of *S. typhi* per mg immunoglobulin in sera of typhoid patients and non-typhoid controls. (o) Anti-LPS antibodies; (•) Anti-protein antibodies.

Table 1. Measurement of serum antibodies to the LPS and protein antigens of *S. typhi* by solid-phase radioimmunoassay in the diagnosis of typhoid fever

		Anti-LPS antibody. No. of sera with titres for:				Anti-protein antibody. No. of sera with titres for:			
Subject	No. of sera examined	Total antibody ≥1,500	IgA-class antibody ≥75	IgG-class antibody ≥750	IgM-class antibody ≥300	Total antibody ≥1,500	IgA-class antibody ≥75	IgG-class antibody ≥750	IgM-c antib ≥3(
Typhoid patients	31	29 (94)*	29 (94)	29 (94)	28 (90)	29 (94)	27 (87)	31 (100)	28 (90
Non-typhoid controls	20	0 (0)	2 (10)	1 (5)	6 (30)	0 (0)	2 (10)	3 (15)	2 (10

* Figures in parentheses indicate percentages relative to the total number of sera examined.

Lack of association between the anti-O agglutination titres and the anti-LPS RIA titres

There was no correlation between the antibody titres measured by bacterial agglutination and thos by SP-RIA against the LPS antigen. The correlation coefficient between the anti-O agglutinatin titres and the total or IgA, IgG and IgM anti-LPS antibody titres were 0·14, 0·33, 0·24 and 0·2(respectively. Among the 14 serum specimens with anti-O agglutination titres below 80, only one hac an anti-LPS RIA titre below 1,500. The remaining 13 all had appreciable amounts of anti-LP antibodies ranging from an RIA titre of 1,600 to 30,000.

The diagnostic value of the RIA was therefore compared with that of the Widal test as shown ir Table 2. When an anti-O or anti-H agglutination titre ≥80 was chosen as the diagnostic level, five o 20 (25%) sera from non-typhoid controls and 25 of 31 (81%) sera from typhoid patients were founc to have antibody titres reaching this level. In contrast, when an anti-LPS or anti-protein RIA titr ≥1,500 was chosen as the diagnostic level, 29 of 31 (94%) sera from typhoid patients but none fron the controls gave a titre reaching this level.

DISCUSSION

The measurement of antibodies to somatic antigens of Gram-negative bacteria in immunized o

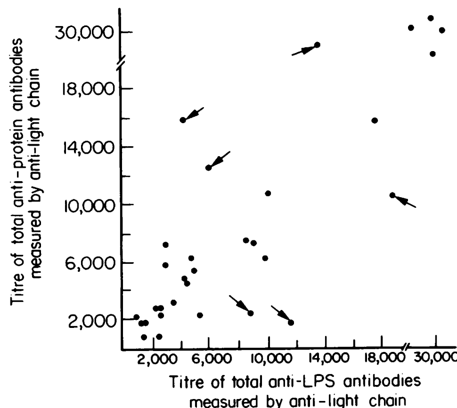

Fig. 4. Scatter diagram showing the relationship between the anti-protein and anti-LPS antibodies in sera o typhoid patients (arrows indicate patients having either high anti-protein but low anti-LPS or high anti-LPS bu low anti-protein antibodies).

Table 2. Measurement of anti-O and anti-H agglutination titres by the Widal test and total anti-LPS and anti-protein antibody titres by radioimmunoassay (RIA) for the diagnosis of typhoid fever

Subject	No. of sera examined	No. of sera with RIA titre ⩾1,500 for total antibodies against: LPS	Protein	No. of sera with Widal titre ⩾80 for antibodies against: O	H
Typhoid patients	31	29 (94)*	29 (94)	17 (55)	25 (81)
Non-typhoid controls	20	0 (0)	0 (0)	1 (5)	5 (25)

* Figures in parentheses indicate percentages relative to the total number of sera examined.

infected animals or in patients has in the past been made mainly by direct or indirect agglutination reactions. However, like other immunological reactions that depend on the secondary manifestation of the primary antigen–antibody binding, these may fail to reveal the true relative quantities of specific antibodies in the different immunoglobulin fractions. Osler, Mulligan & Rodriguez (1966) reported that 7S IgG antibodies, particularly those formed during the early phase of the immune response, might not show agglutinating capacity. Therefore, studies done in the past to measure the type of antibody response to enterobacterial somatic antigen by agglutination might have underestimated the amount of 7S IgG antibodies formed against it, and might thus have led to the conclusion of an exclusive IgM antibody response to somatic antigen (Lospalluto *et al.*, 1962; Fink *et al.*, 1962; Bauer, Mathies & Stavitsky, 1963). To provide real answers to these types of problems, a method to measure all antibody molecules regardless of their agglutinating, precipitating or complement-fixing properties is needed; one such method is RIA based on the primary function of all antibodies, i.e. specific binding to antigens. In our SP-RIA system, although we could not compare the relative amounts of specific antibodies in the different immunoglobulin classes since the titres of total, IgA, IgG and IgM antibodies obtained were dependent on the binding of different radiolabelled secondary antibodies to the respective primary antibodies in patients' sera, we were able to demonstrate clearly that humoral antibodies to LPS antigen in typhoid patients were not confined to the IgM immunoglobulin class but were also present in the IgA and IgG immunoglobulin classes at a level significantly higher than the normal controls (Figs 2 & 3).

The Widal agglutination reaction was found unsatisfactory for the serological diagnosis of typhoid fever in endemic areas (Levine *et al.*, 1978). Patients with the same anti-O agglutinating titre might have considerable differences in the amount of anti-LPS antibodies. There was clearly no close correlation between the anti-O agglutinating titres and the anti-LPS RIA antibody titres (as measured by anti-light chain antibodies).

The importance of Salmonella protein antigens in eliciting protective immunity in experimental animals has been demonstrated by several groups of workers (Barber & Eylan, 1975; Plant *et al.*, 1978; Kuusi *et al.*, 1979). In the present study, it was found that in typhoid patients a good positive correlation existed between the antibody response to these two types of antigens. However, a divergent antibody response to the LPS and protein antigens in a few of the patients indicated the non-identity of these two antigens.

The possibility of developing better methods for the serological diagnosis of typhoid fever has also been explored. The best differentiation of typhoid patients from non-typhoid subjects was achieved by measuring the total antibody titre to either the protein or the LPS antigens. Ninety-four per cent of typhoid patients had anti-LPS or anti-protein antibody titres ⩾1,500 as measured by anti-light chain, while none of the 20 normal controls had similar titres. In this respect, RIA was superior to the Widal test in the diagnosis of typhoid fever. Since RIA involves the use of expensive instruments and hazardous radioactive reagents, for routine use it should be modified as an ELISA assay.

This investigation was assisted financially by a grant to P.Y.C. from the Diarrhoeal Disease Control Programme of the World Health Organization.

REFERENCES

BARBER, C. (1961) Le tampon-veronal, moyen d'extraction des antigens bacteriens. *Arch. Roum. Pathol. exp. Microbiol.* **20,** 541.

BARBER, C. & EYLAN, E. (1974) Are all the Salmonella serological O factors polysaccharides? *Microbios*, **9,** 173.

BARBER, C. & EYLAN, E. (1975) Confirmation of the protective role of proteins from *S. typhimurium* in infection of mice with their natural pathogen. *Zentralbl. Bakteriol. Parasitenkund. Abt. I (Origa.)*, **230,** 461.

BARBER, C. & EYLAN, E. (1976) The unfortunate role of precedent in bacteriology. I. The main antigens of Salmonellae: the proteins. *Zentralbl. Bakteriol. Parasitenkund. Abt. I (Orig. A)*, **234,** 53.

BAUER, D.C., MATHIES, M.J. & STAVITSKY, A.B. (1963) Sequence of synthesis of γ-1 macroglobulin and γ-2 globulin antibodies during primary and secondary responses to proteins, Salmonella antigens and phage. *J. exp. Med.* **117,** 889.

CHERNOKHVOSTOVA, E., LUXEMBURG, K.I., STARSHINOVA, V., ANDREEVA, N. & GERMAN, G. (1969) Study on the production of IgG-, IgA- and IgM-antibodies to somatic antigens of *Salmonella typhi* in humans. *Clin. exp. Immunol.* **4,** 407.

FINK, C.W., MILLER, W.E., JR., DORWARD, B. & LOSPALLUTO, L. (1962) The formation of macroglobulin antibodies. II. Studies on neonatal infants and older children. *J. clin. Invest.* **41,** 1422.

FUKAZAWA, Y., SHINODA, T., YOMODA, T. & TSUCHIYE, T. (1970) Antibody response to bacterial antigens; characteristics of antibody response to somatic antigens of *S. typhimurium. Infect. Immun.* **1,** 219.

GANNON, P.J., SURGALLA, M.J., FITZPATRICK, J.E. & NETER, E. (1980) Immunoglobulin G and immunoglobulin M antibody response of patients with malignancies to the O antigens of bacteria causing bacteremia. *J. clin. Microbiol.* **12,** 60.

JAWETZ, E., MELNICK, J.L. & ADELBERG, E.A. (1978) *Review of Medical Microbiology* 13th edn, p. 216. Lange Medical Publications, Los Altos, California.

KUMAR, K., MALAVIYA, A.N., MURTHY, R.G.S., VENKATARAMAN, M. & MOHAPATRA, L.N. (1974) Immunological study of typhoid: immunoglobulins, C3, antibodies and leukocyte migration inhibition in patients with typhoid fever and TAB-vaccinated individuals. *Infect. Immun.* **10,** 1219.

KUUSI, N., NURMINEN, M., SAXEN, H., VALTONEN, M. & MAKELA, P.H. (1979) Immunization with major outer membrane proteins in experimental salmonellosis of mice. *Infect. Immun.* **25,** 857.

LA BROOY, J.T., ROWLEY, D. & SHEARMAN, D.J.C. (1980) Measurement of intestinal antibody by radioimmunoassay. *Clin. exp. Immunol.* **41,** 281.

LEVINE, M.M., GRADOS, O., GILMAN, R.H., WOODWARD, W.E., SOLIS-PLAZA, R. & WALDMAN, W. (1978) Diagnostic value of the Widal test in areas endemic for typhoid fever. *Am. J. Trop. Med. Hyg.* **27,** 795.

LOSPALLUTO, J., MILLER, W., JR, DORWARD, B. & FINK, C.W. (1962) The formation of macroglobulin antibodies. I. Studies on adult humans. *J. Clin. Invest.* **41,** 1415.

MANCINI, G., VAERMAN, J.P., CARBONARA, A.D. & HEREMANS, J.F. (1964) A single radial-diffusion method for the immunological quantitation of proteins. In *Protides of the Biological Fluids* (ed. by H. Paeters), pp. 370–376. Elsevier North-Holland, Amsterdam.

OSLER, A.G., MULLIGAN, J.J., JR. & RODRIGUEZ, E. (1966) Weight estimates of rabbit anti-human serum albumin based on antigen-binding and precipitin analyses: specific haemagglutinating activities of 7S and 19S components. *J. Immunol.* **96,** 334.

PLANT, J., GLYNN, A.A. & WILSON, B.M. (1978) Protective effects of a supernatant factor from *Salmonella typhimurium* on *Salmonella typhimurium* infection of inbred mice. *Infect. Immun.* **22,** 125.

ROSSEN, R.D., WOLFF, S.M. & BUTLER, W.T. (1967) The antibody response in nasal washings and serum to *S. typhosa* endotoxin administered intravenously. *J. Immunol.* **99,** 246.

TURNER, M.W. & ROWE, D.S. (1964) Characterization of human antibodies to *Salmonella typhi* by gel filtration and antigenic analysis. *Immunology*, **7,** 639.

WEIDANZ, W.P., JACKSON, A.L. & LANDY, M. (1964) Some aspects of the antibody response of rabbits to immunization with enterobacterial somatic antigens. *Proc. Soc. exp. Biol. Med.* **116,** 832.

WESTPHAL, O. & JANN, K. (1965) In *Methods in Carbohydrate Chemistry* (ed. by R. L. Whistler), Vol, 5, pp. 83–91. Academic Press, New York.

ZOLLINGER, W.D., DALRYMPLE, J.M. & ARTENSTEIN, M.S. (1976) Analysis of parameters affecting the solid phase radioimmunoassay quantitation of antibody to meningococcal antigens. *J. Immunol.* **117,** 1788.