Clin. exp. Immunol. (1980) **41**, 281–289.

Measurement of intestinal antibody by radioimmunoassay

J. T. LA BROOY,* D. ROWLEY† & D. J. C. SHEARMAN* *Departments of *Medicine, and †Microbiology and Immunology, University of Adelaide, South Australia*

(Accepted for publication 25 January 1980)

SUMMARY

The study of antibody responses in the intestine has been greatly hampered by lack of reproducible sensitive assays. An assay for measuring antibody against bacteria capable of regularly detecting gut antibody in gastroenteritis is described. It is based on absorption of antibody onto bacteria and measurement of the amount of antibody bound using radiolabelled anti-immunoglobulin antibody. Anti-light chain antibody is used to detect all classes of antibody as well as partially degraded antibody which retains the capacity to bind; anti-alpha and anti-gamma antibody is used to measure IgA and IgG antibody. The sensitivity of the assay depends on the use of anti-immunoglobulin antibody purified by affinity chromatography and allows measurement of nanogram amounts of antibody. Its specificity and kinetics are described and the particular advantages it provides in the measurement of antibacterial antibody in the intestine are discussed.

INTRODUCTION

The dissociation that may occur between secretory and serum antibody responses to antigens at secretory surfaces (Burrows, Elliott & Havens, 1947; Smith *et al.*, 1966) makes attempts at assessing intestinal immunity through measurement of serum antibody of limited use. Yet the difficulties in measuring local immune responses in the intestine have obliged most workers in the field of enteric immunity to continue with serum antibody measurements for many of their studies on protective immunity.

Of these difficulties, lack of a satisfactory assay for intestinal immunity is the most important (Shearman, Parkin & McClelland, 1972). The development of such an assay was a prerequisite for work on bacterial gastroenteritis. The major constraint on an assay for this purpose is that of being able to measure antibody in the proteolytic intestinal fluid. The degree of proteolysis of immunoglobulin varies at different sites in the gut. Gastric juice and faeces are unproductive as sources of intestinal antibody (Samson, McClelland & Shearman, 1973; Freter, 1962) while duodenal and jejunal fluid have been more useful in this regard. In the alkaline tryptic environment of the small intestine, the predominant immunoglobulin, secretory IgA, remains intact. Monomeric IgA, IgG and IgM are partially degraded but the fragments appear to retain their primary property of specific binding (Brown, Newcomb & Ishizaka, 1970; Samson *et al.*, 1973). The published evidence suggests that attempts to inhibit proteolysis by using enzyme inhibitors such as Trasylol, or by heating intestinal fluid to 56°C to denature the enzymes, are no more useful as a preliminary to detecting antibody than rapid freezing of the intestinal fluid (Samson *et al.*, 1973; Horsfall & Rowley, 1979). Fortunately the evidence indicates that intestinal antibody functions through its primary binding

Correspondence: Dr J. T. La Brooy, Department of Medicine, Royal Adelaide Hospital, Adelaide, South Australia 5000.

0099-9104/80/0800-0281$02.00

ability by interfering with bacterial adherence to gut epithelium (Freter, 1969; Steele, Jenkin & Rowley, 1977). An assay dependent on primary binding of the antibody should be useful and need not involve the Fc-mediated functions of antibody. This statement is supported by the finding that in an animal model of cholera, the protective ability of antibody fragments was comparable to that of intact antibody (Steele, Chaicumpa & Rowley, 1975).

For these reasons our main thrust was in the development of a sensitive assay that depended on the ability of antibody to bind antigen. An additional but less important attribute of such an ideal assay was the ability to determine the distribution of antibody in the different immunoglobulin classes, thus increasing its value in analysing the intestinal antibody response.

An assay that potentially met these requirements was described by Nielson, Parratt & White (1973) for detecting antibody to *Micropolyspora faeni*, the aetiological agent in 'farmer's lung'. This paper is concerned with the validation of this method for measuring intestinal antibody. The results obtained through its application to the study of bacterial gastroenteritis is the subject of the accompanying communication (La Brooy *et al.*, 1980).

MATERIALS AND METHODS

Immunoglobulins. IgA was prepared from the serum of a patient with IgA myeloma using gel chromatography on Sephadex G-200. The 7S peak was separated further on DE52 cellulose. The IgG was washed through with 0·02 M sodium hydrogen phosphate at pH 6·3 and the IgA eluted with 0·05 M sodium chloride in 0·2 M sodium hydrogen phosphate, pH 6·3.

Secretory IgA was prepared from pooled human colostrum (Newcomb, Normansell & Stanworth, 1968).

IgG was purified from an ammonium sulphate cut of normal serum via DEAE52 ion exchange chromatography.

Light chains were prepared from IgG using reductive cleavage with mercaptoethanol and separation of the polypeptide chains on Sephadex G-75 in 1 N acetic acid (Fleischman, Pain & Porter, 1961).

The purity of these preparations was checked by immunoelectrophoresis, Ouchterlony analysis, sodium dodecyl sulphate, polyacrylamide gel electrophoresis and Mancini single radial immunodiffusion. Commercial (Behringwerke) antisera against alpha, gamma and mu heavy chains, secretory component and whole serum were used for these analyses.

Antisera. Antisera against IgA and IgG were raised in goats. Five milligrams of immunoglobulin in Freund's complete adjuvant were injected into multiple subcutaneous sites and this was followed by monthly injections of 2 mg immunoglobulin in Freund's incomplete adjuvant. Serum was harvested after 9 months.

Affinity chromatography was used to further purify these antisera and to obtain specific antibodies to alpha, gamma and light chains. Affinity columns of Sepharose 4B linked to secretory IgA, IgG and light chains were prepared using the purified immunoglobulins and commercial (Pharmacia) cyanogen bromide-activated Sepharose. The antisera were applied to these columns in 0·1 M phosphate buffer (pH 7·4) and eluted with 3 M sodium thiocyanate.

The purified anti-IgA eluted off the secretory IgA column was passed through an IgG affinity column to remove light chain cross-reactivity. The anti-gamma antibody was similarly obtained by purifying anti-IgG on the IgG affinity column and removing light chain cross-reactivity on the light chain column. The anti-light chain antibody was eluted off the light chain column after the passage of anti-IgG through that column. These antibody preparations were concentrated to 2 mg/ml and their specificity demonstrated using immunoelectrophoresis and Ouchterlony analysis.

Radioiodination. The purified antibodies were iodinated with ^{125}I (Amersham) by the method of Hunter & Greenwood (1962). The free iodine was removed by passage through a Sephadex G-25 column. The specific activity of the antibodies after various labellings was between 1,000 and 4,000 c.p.m./ng of antibody and they were used for 2 months after labelling. Radiolabelled antibody was stored at 4°C in 0·1% bovine serum albumin (BSA) and 0·1% sodium azide.

Bacteria. These were grown from single colonies in 1-litre volumes of nutrient broth at 37°C

overnight on a shaker. After three washes in normal saline the bacteria were fixed with 0·2% glutaraldehyde for 30 min and thoroughly washed in three changes of saline before storing at a concentration of 10^{11}/ml in phosphate-buffered saline.

Bacterial lipopolysaccharide (LPS). The LPS used in the approximate quantitation of the assay was obtained from *Salmonella bovis-morbificans*, using the phenol/water extraction technique described by Westphal, Luderitz & Bister (1952).

Standard assay procedure. Plastic tubes (1 × 7·5 cm) were used in the assay. Phosphate-buffered saline (0·04 M phosphate, pH 7·4, 0·15 M sodium chloride) containing 0·05% bovine serum albumin (PBS·BSA) was used for all dilutions and washings in the assay which were done in the cold throughout.

Doubling dilutions of the sample were made in 0·1-ml volumes in the tubes. The freshly washed bacteria were used at 10^{10}/ml in PBS·BSA. Fifty-microlitres of this suspension was added to each dilution, mixed thoroughly and left on ice for 30 min. Any unbound immunoglobulin was then removed from the bacteria by four washes, each with 2·5 ml of PBS·BSA, centrifuging at 2,000 r.p.m. for 15 min between each wash. Radiolabelled antihuman immunoglobulin antibody (5–20 ng and around 10,000 c.p.m.) was added to the bacterial pellet in a volume of 25 μl diluent and allowed to react with the antibacterial antibody for 60 min. (The antibacterial antibody will be referred to as primary antibody, PAb, and the anti-immunoglobulin antibody as secondary antibody, SAb.) Unbound secondary antibody was removed during three more wash-and-centrifuge cycles. The radioactivity bound to the bacterial pellets was counted in a gamma counter.

The amount of radioactivity bound was plotted against the dilution of primary antibody on semilog paper giving a curve with three zones (Fig. 1) corresponding to a plateau of maximum binding of secondary antibody, a zone of decreasing binding, levelling off to the third zone of background non-specific binding in those tubes where the primary antibody had been diluted out to negligible levels. The antibody titre of the fluid was taken as the dilution at which 10% of the added radioactive secondary antibody was bound.

Anti-light chain antibody was used as secondary antibody for measuring total primary antibody irrespective of immunoglobulin class, anti-alpha chain antibody for measuring IgA antibody and anti-gamma antibody for measuring IgG antibody.

Immune intestinal fluid and sera. Intestinal fluid and sera from patients recovering from Salmonella and Shigella gastroenteritis were assayed with the autologous pathogens. A double-lumen tube weighted with a mercury bag was used to aspirate intestinal fluid from beyond the ligament of Treitz, as checked by fluoroscopy.

RESULTS

Number of washes

A prozone effect with lower dilutions of primary antibody was seen if the number of washes was inadequate to remove non-specific immunoglobulin. Four washes prior to adding secondary antibody appeared optimal for the assay.

Effects of temperature

Roughly twice as much intestinal antibody was bound at 4°C than at 20°C. This phenomenon was not demonstrable with serum antibody or with secondary antibody. It possibly represents a low thermal amplitude of intestinal antibody.

Time

Equilibrium appeared to have been reached within 30 min of incubating bacteria with the primary antibody. The 60 min allowed for the second step was, however, a compromise since several hours were required for equilibration with secondary antibody in the amounts used.

Bacteria as the absorbent antigenic surface

A basic requirement was that the assay be conducted in antigen excess. That this held true was

shown by the addition of five-fold less bacteria than usual with no resultant decrease in binding of radioactive secondary antibody.

The antibodies being measured in the assay were mainly against lipopolysaccharides and other stable antigens. This was shown by comparing the results using bacteria inactivated by various means. Ethanol fixation removed or destroyed some of the antigens since the suspension, although in excess, could not adsorb as much antibody as bacteria inactivated by heat or glutaraldehyde (Fig. 1).

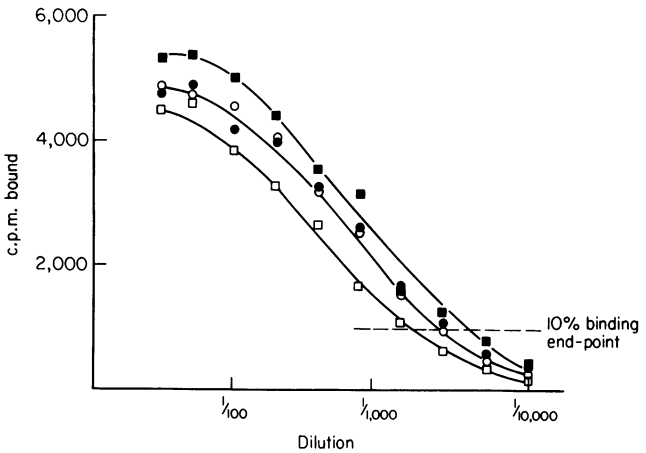

Fig. 1. The effect of preparing bacteria in different ways for use as immunoadsorbent gives rise to differences in binding of antibody in the assay. (●) Live bacteria (titre 1/3,000); (■) glutaraldehyde-fixed bacteria (titre (1/3,500); (○) boiled bacteria (titre 1/3,000); (□) ethanol-fixed bacteria (titre 1/1,800).

Volumes of antibody-containing fluid

Increasing the volume of the initial fluid sample (PAb) proportionately increased the amount of antibody bound to the bacteria. This emphasized that antigen was in excess during the assay. For convenience 0·1-ml volumes were used.

Radiolabelled secondary antibody

The maximum binding of radiolabelled secondary antibody achieved using the assay was 50–60% of the total added to the complex of primary antibody and bacteria. That this was not because of damage to the secondary antibody during elution off the affinity column or to damage during iodination, was demonstrated by over 95% binding of the radiolabelled antibody on re-exposure to the appropriate Sepharose 4B-linked immunoglobulins. Studies of the binding of both anti-alpha and anti-light chain antibody to human serum insolubilized by glutaraldehyde (Avrameas & Ternynck, 1960) and then incubated with secondary antibody and washed as in the assay, gave 50–60% binding. The reasons for this suboptimal binding under the conditions of the assay remain uncertain. It may reflect disruption of antibody by the physical forces generated during centrifuging or it may be that some of the antigenic sites on the primary antibody against which the secondary antibody is directed are masked when antibody binds to antigen or is insolubilized with glutaraldehyde.

Under the conditions of the assay described the primary antibody was not saturated with secondary antibody, although saturation could be achieved by greatly increasing the amount of secondary antibody added (Fig. 2).

The amount of secondary antibody bound to primary antibody on the bacteria was directly related to the amount added over a wide range of concentrations (Fig. 3). This allowed the antibody titre to be taken as the dilution at which 10% of the added radioactivity remained bound to the bacterial pellet. This end-point was constant for the amounts of secondary antibody added (varying

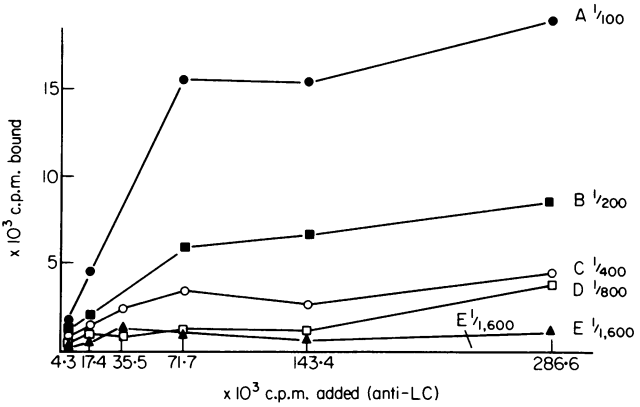

Fig. 2. Saturation of primary antibody by secondary antibody. A–E represent dilutions of an immune serum to which increasing amounts of secondary antibody were added. The primary antibody bound to the bacteria could be saturated with secondary antibody as demonstrated by the plateau obtained with greatly increased amounts of secondary antibody.

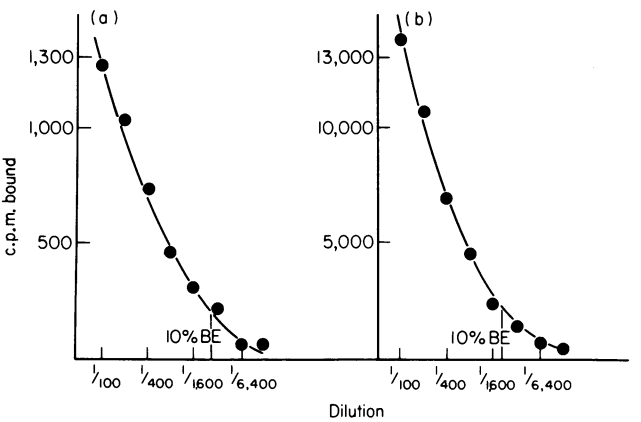

Fig. 3. There was a ten-fold difference in secondary antibody added to these paired series of dilutions of primary antibody: (a) 10 ng (2,000 c.p.m.) and (b) 100 ng (20,000 c.p.m.). The proportion of secondary antibody bound/secondary antibody added remained constant at the levels of secondary antibody used.

in the range of 5–20 ng of secondary antibody) and it was well above the 0·2–2·0% non-specific background binding seen when secondary antibody was added to bacteria not exposed to anti-bacterial antibody.

Variation intra-assay and inter-assay

The reciprocal of the titres estimated on eight identical series of a serum gave a mean of 1,637 with a scatter between 1,400 and 1,900 and a standard deviation of 210.

Inter-assay variation was estimated from the titres of aliquots of an immune serum measured in thirteen consecutive assays. This gave a mean titre of 1,233 with a standard deviation of 429 and a scatter of 680 to 2,150.

To reduce inter-assay variation, two series of an immune serum with a titre of 1/1,000 were run in each assay and the other assays were standardized by the results obtained.

Specificity of assay

The specificity of the assay was demonstrated by using organisms of differing antigenic relationships in the primary absorption step. Specificity within even the same bacterial genus is shown in Fig. 4 by the binding of antibody from the serum of a patient with *Salmonella typhimurium* gastroenteritis to *Salmonella typhimurium*, *Salmonella newport*, *Salmonella senftenberg*, *Salmonella adelaide* and to Citrobacter. According to the Kauffmann–White scheme (Kauffmann, 1975) *Salmonella typhimurium* ('0' 1, 4, 5 and 12) shares two major antigens with Citrobacter ('0' 4 and 5) (Jenkin & Rowley, 1965) and one with *Salmonella senftenberg* ('0' 1). There are no identified cross-reactions between *S. typhimurium* and *S. newport* or *S. adelaide* but they no doubt share minor core antigens.

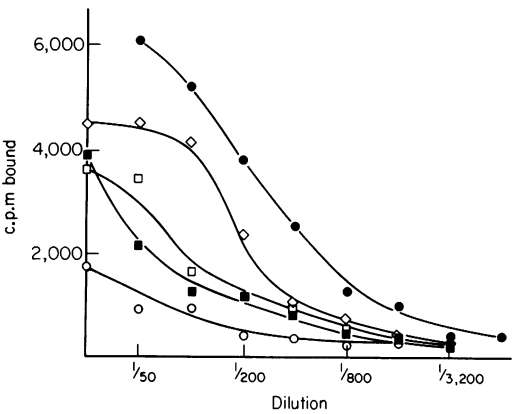

Fig. 4. Binding curves obtained on titration of serum from a patient recovering from *Salmonella typhimurium* ('0' antigens 1, 4, 5, 12 and 'H' antigens i: 1, 2) against *Salmonella typhimurium* (●), *Salmonella senftenberg* ('0' 1, 3, 19; 'H' g (s) t) (■), *Salmonella newport* ('0' 35; 'H' f, g) (□), *Salmonella adelaide* ('0' 6, 8; 'H' eh: 1, 2) (○) and Citrobacter ('0' 4, 5) (◇).

Natural antibody or non-specific binding

At low dilutions, serum from patients not known to have had an infection with the organisms showed binding of antibody. That this was due to specific antibody was indicated by reduction in titre after bacterial absorption with no measurable change in total immunoglobulin concentration (Fig. 5). Presumably this represents cross-reacting antibody formed against indigenous bacterial flora or following previous infections.

Quantitation of the assay

Using a high titre serum from a patient with *Salmonella bovis-morbificans* infection, an attempt was made to convert this titre into weight of antibody. For this purpose paired 2-ml aliquots of the serum were absorbed in the cold for 48 hr with 2 and 4 mg respectively of protein-free lipopolysaccharide (LPS) of *Salmonella bovis-morbificans*. The LPS and antibody attached to it were spun out on the Spinco at 100,000 ***g*** for 1 hr. This antigen–antibody complex was washed well with cold saline, re-centrifuged and the bound protein determined by Folin analysis (Lowry *et al.*, 1951). On assaying the original serum it had a titre of 7,900 units/0·1 ml (using anti-LC as SAb) and 98% of this activity was absorbed out. The antibody protein bound to the LPS was 3,000 μg from each of the 2-ml aliquots of serum. This indicated that the patient's serum contained 1,500 μg of antibody/ml. With the assay titre of 7,900 units/0·1 ml, this means that 1 dilution unit is equivalent to 20 ng of antibody.

The assay of antibody in intestinal fluid

That intestinal fluid interfered with the measurement of antibody by this assay seemed unlikely in

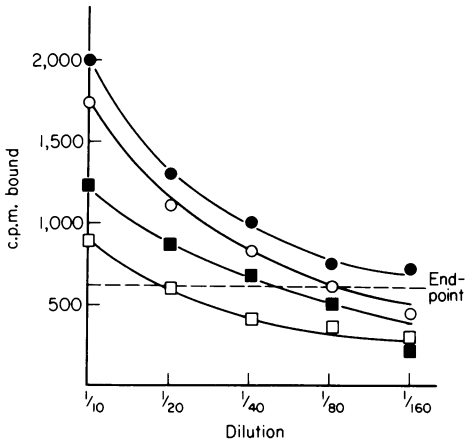

Fig. 5. Reduction of low levels of binding seen in the serum of a child with no history of exposure to *Salmonella typhimurium* by *Salmonella typhimurium* (10^{10} bacteria) added for 1 hr to 5 ml of a 1 in 10 dilution of the serum during each absorption. (●——●) Preabsorption, (○——○) after one absorption, (■——■) after two absorptions, (□——□) after three absorptions.

view of the results obtained when checking specificity, reproducibility and kinetics of the assay with immune intestinal fluids (see above).

To investigate this further, an ammonium sulphate precipitation (with 0·5% normal goat serum as a carrier protein), of globulin from two freshly thawed immune intestinal fluids was performed. The protein precipitated was washed, dissolved in saline and dialysed. The titres of antibody assayed in saline were identical with the titres in duplicate samples of untreated intestinal fluid, suggesting that there was no interference by the intestinal fluid with the assay system. Furthermore, on seeding pooled normal intestinal fluid diluted 1 in 10 with a hundredth of its volume of an immune serum, there was no difference in the antibody titre of the immune serum assayed in the milieu of the intestinal fluid compared to its titre in saline.

Detection of antibody of different immunoglobulin classes

The mono-specificity of the anti-alpha and anti-gamma secondary antibodies at a concentration of 2 mg/ml was demonstrated by immunoelectrophoresis and Ouchterlony analysis using whole human serum, SIgA and IgG. The clarity with which the labelled secondary antibodies distinguished primary antibodies of IgA and IgG classes was further demonstrated in the assay itself by inhibition.

Fifty nanograms of pure secretory IgA, added at the time of the second incubation step, inhibited binding of the anti-alpha antibody to the complex of bacteria and primary antibody near its limiting dilution for IgA antibody, whereas 20,000 ng of IgG failed to do this. Similiarly, 13 ng of IgG inhibited binding of anti-gamma antibody to the complex of bacteria with primary antibody near its limiting dilution for IgG antibody, while 20,000 ng of secretory IgA could not do this.

This high degree of specificity was reflected by the results in practice where there was vastly more IgG than IgA antibody in serum and considerably more IgA antibody than IgG in secretions.

DISCUSSION

Most previous attempts at measuring antibody in intestinal fluid used bacterial agglutination or haemagglutination of red cells coated with bacterial antigen. These techniques require cross-linking, a function of the divalency of antibody, and therefore fail to detect monomeric binding fragments of antibody. Furthermore, their use has been marked by poor reproducibility (Freter,

1962; Waldman *et al.*, 1972) due partly to non-specific agglutination. With the advent of radioimmunoassay, more effective studies of intestinal antibody responses have become possible. Freter used the Farr assay for this purpose with *V. cholerae* lipopolysaccharide (Freter, 1962; Freter *et al.*, 1965). Though he obtained meaningful results the sensitivity was low. Work in our laboratory has shown that other bacterial lipopolysaccharides are precipitated to a variable extent with ammonium sulphate and this would greatly complicate the use of the Farr assay in other bacterial infections (Horsfall & Rowley, 1979). Waldman and co-workers developed an assay similar to the one described here for measuring intestinal antibody responses in subjects with cholera (Waldman *et al.*, 1972). In their studies, fluids were lyophilized and reconstituted in a concentrated form before being assayed and only general conclusions could be drawn. In summary, it would be true to say that there is no accepted sensitive assay for measuring antibacterial antibody directly in intestinal fluid.

The assay described in this paper overcomes some of the inadequacies of previous assays for measuring intestinal antibody to bacteria. Its ability to measure all antibody and antibody fragments dependent only on their capacity to bind to antigen is a major advantage in view of the proteolytic damage sustained by antibody in intestinal fluid. This facility depends on the use of the radiolabelled anti-light chain antibody to provide a uniform probe for all classes of immunoglobulin.

The sensitivity of this assay is clearly greater than that provided by assays depending on agglutination or the Farr assay (Horsfall & Rowley, 1979) and it was adequate for the purpose for which it was adapted (see La Brooy *et al.*, 1980). For this degree of sensitivity purification by affinity chromatography of the secondary anti-immunoglobulin antibody was essential. Increasing the volume of intestinal fluid in the primary incubation step could potentially increase the sensitivity of the assay further.

Proteolysis did not prove as much of a problem as had been anticipated with this assay. The use of anti-light chain antibody and the nature of proteolysis in a predominantly tryptic environment no doubt contributed to this. The bacteria themselves and the lipopolysaccharide, which appears to be the most dominant antigen, are resistant to proteolysis. The limited time of exposure of the antibody after thawing the intestinal fluid and the low temperature throughout this assay would limit the enzymatic damage.

Technical limitations of the assay as described here were that the second antigen–antibody reaction was terminated before equilibrium was reached and that the amounts of secondary antibody were not sufficient to saturate the primary antibody bound in the bacteria–antibody complex. The variability introduced is decreased by using a standard, or ideally, a panel of standards to improve comparison between samples analysed in different assay runs. The quantitation of antibody by this assay will require more determinations of antibody in sera by other methods before its accuracy can be asserted with confidence. Similarly, comparisons of relative amounts of antibody in different immunoglobulin classes can only be regarded as approximate in view of the fact that the titres are expressed in terms of the binding of different secondary antibodies. This did not prove a great limitation in practice.

This assay has the potential of being applied to other antigens against which intestinal antibodies may be directed, provided the antigen can be used in a stable, insolubilized form. The importance of the form in which the antigen is presented should be apparent from the results of the ethanol fixation of bacteria in this assay.

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