INFECTION AND IMMUNITY, May 1992, p. 2023–2029
0019-9567/92/052023-07$02.00/0

Vol. 60, No. 5

Indirect Measurement of Intestinal Immune Responses to an Orally Administered Attenuated Bacterial Vaccine

BRUCE D. FORREST†

Department of Medicine, Royal Adelaide Hospital, Adelaide, Australia

Received 9 September 1991/Accepted 28 February 1992

Intestinal fluid, saliva, circulating peripheral blood lymphocytes (PBL), and serum samples obtained from 81 human adult subjects who had been orally vaccinated with either *Salmonella typhi* Ty21a or one of its recombinant derivatives were examined to determine the value of indirect measurements of an antigen-specific intestinal-immunoglobulin A (IgA) response. Salivary IgA failed to provide consistent or correlative responses, and no evidence of a significant relationship was apparent with the intestinal-IgA responses. No significant correlation between the specific increase in responses in serum IgA and intestinal IgA was evident. While the magnitude of the serum IgG response significantly correlated with the intestinal-IgA response ($P = 0.00064$), it failed to detect 14.8% of the intestinal-IgA responses. The observation that 16.6% of the subjects had delayed serum IgA responses, with a peak occurring after day 23 compared with days 12 to 14, may have contributed to the inadequacy of the serum IgA response as a correlative indicator. The serum IgG responses in these subjects were also of a diminished magnitude. Specific IgA production by circulating PBL was found to be the most sensitive (92.6% response rate) and correlative ($P = 0.00071$) indicator of a specific intestinal-IgA immune response. However, its value in predicting protective efficacy is untried. These studies confirm that for the assessment of an enteric bacterial vaccine, determination of in vitro specific IgA production by circulating PBL may offer a single measurement of specific immunity which is as useful as serum and intestinal measurements combined.

The development of effective vaccines against infectious diseases requires the existence of reliable and meaningful immunological correlates of protection. For enteric infectious diseases, it is widely accepted that the induction of strong local intestinal immune responses, especially of the secretory immunoglobulin A (IgA) class, is necessary for the efficacy of such vaccines (41, 42). However, assessment of the effectiveness of orally administered enteric vaccines in the induction of local immunity has instead frequently relied on indirect measurements of intestinal antibody, such as determination of levels of specific antibody in serum (2, 26, 28, 32), colostrum, or saliva (20, 21), and more recently has relied on determination of specific antibody production by circulating peripheral blood lymphocytes (PBL) (8, 11, 19, 22, 23). Indirect measurements do not necessarily reflect intestinal immune responses accurately (7, 12, 20, 21), and so, to measure local immune responses to orally administered vaccines, developers have resorted to using either saline purges (20, 37, 38) or intestinal intubations with direct sampling of jejunal fluid (9, 12–15, 24, 25, 27, 29, 36, 39).

The continued inability to agree on a reproducible method or to have confidence in an existing method for the determination of specific intestinal-IgA immune responses presents a significant obstacle to the development of an enteric vaccine. Broadly speaking, the only reliable method at present for the confident prediction of the probable effectiveness of an enteric vaccine is through volunteer challenge studies that use the actual pathogen, which would result in a largely unsatisfactory situation of uncertain ethics. This paper presents a comprehensive series of studies which have attempted to address several important issues in the assessment of enteric vaccines through assessing certain indirect measurements, such as the production of specific antibody in serum, saliva, and PBL, as correlates of specific antibody in intestinal fluid.

MATERIALS AND METHODS

Vaccine strains, doses, and administration. The vaccine strain used in these studies was either the live orally administered typhoid vaccine strain *Salmonella typhi* Ty21a, an attenuated Vi antigen-negative mutant of the pathogenic strain *S. typhi* Ty2 (16, 18), or one of its recombinant derivatives (EX210, EX645, or EX363) that also carried and expressed the genes for *Vibrio cholerae* O antigen. The construction and characterization of the vaccine strains EX645 and EX363 have been described fully elsewhere (10, 11, 39). All vaccine doses were supplied by Enterovax Limited, Salisbury, Australia, as individual doses. Each vaccine dose consisted of between 5.2×10^{10} and 1.8×10^{11} viable organisms (mean total number) confirmed by colony counts. Each vaccine dose was orally administered according to a standard vaccination schedule of three alternate daily doses (9, 12–14, 39). Subjects fasted for 8 h prior to vaccination. The vaccine doses were suspended in 40 ml of 0.9% saline and swallowed 5 to 10 min after the ingestion of 50 ml of a 2% sodium bicarbonate solution. This pretreatment was necessary to neutralize the gastric acid of the subjects. Gastric acid has been demonstrated to have an adverse effect on the viability of live orally administered enteric organisms (17); in addition, it is able to alter the immunogenicity of inactivated oral vaccine preparations (6).

Subjects and data analysis. Data from 81 orally vaccinated adult subjects (aged 18 to 50 years) were available. A total of 46 subjects had received doses containing $\approx 10^{11}$ viable *S. typhi* Ty21a organisms, and 35 subjects had received doses containing $\approx 10^{11}$ viable EX210, EX645, or EX363 organ-

† Present address: Department of Medical Microbiology, Division of Communicable Diseases, Royal Free Hospital, Pond Street, London NW3 2QG, United Kingdom.

TABLE 1. Subjects and sample collections

Sample[a]	No. of subjects from each group[b]			Total no. of subjects
	A	B	C	
Intestinal fluid	14	22	45	81
Serum				
Serial	14	22		36
Days 0 and 12 or 14			45	45
Saliva		22		22
PBL	14	22	45	81

[a] Intestinal fluid was collected prevaccination (day 0) and postvaccination either on day 14 or 15 or on both days 14 or 15 and 21. Serum was collected prevaccination (day 0) and either serially every 3 to 4 days postvaccination (groups A and B) or as a single postvaccination sample obtained on day 14 or 15 concurrently with intestinal fluid (group C). Saliva was collected from group B subjects concurrently with intestinal fluid. PBL were collected from all subjects (groups A, B, and C) prevaccination (day 0) and on day 7 postvaccination.

[b] Subject groups were determined according to the nature of the samples obtained rather than the vaccine administered.

isms. All of the subjects received the oral vaccines through their participation in a series of clinical studies performed at the Department of Medicine of the Royal Adelaide Hospital, Adelaide, Australia. Written and informed consent had been obtained from all subjects prior to their entry into any of the studies from which this data was obtained. Approval for the use of human subjects was granted by the Human Ethics Committee of the Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide. None of the subjects had any known previous exposure to typhoid fever through vaccination or disease. None of the subjects had any history or current symptoms of gastrointestinal tract disease at the time of participation.

Analysis of the data for each vaccine organism failed to identify any significant differences between the mean increases in specific antityphoid lipopolysaccharide (LPS) IgA antibody of *S. typhi* Ty21a and its hybrid recombinant derivatives in intestinal fluid ($P = 0.36$), in serum ($P = 0.23$), or produced by PBL ($P = 0.65$) or serum IgG ($P = 0.67$). In view of these observations, it was considered appropriate that the two datum collections be pooled. While intestinal immune response data for all 81 subjects were available, the availability and nature of other samples did vary and are detailed in Table 1. The subjects were allocated to three groups, which are detailed in Table 1, only for the purpose of identifying the nature of the samples obtained and not according to the nature of the vaccines administered.

Collection of samples. Intestinal-fluid samples were obtained directly from the upper jejunum by using an ANPRO AN20 Andersen tungsten-weighted sump tube (H. W. Andersen Products, Oyster Bay, N.Y.). In all subjects, correct positioning of the intestinal tube was confirmed by fluoroscopy (36). The use of fluoroscopy for this purpose was restricted by the Ethics Committees, which limited the number of occasions that the procedure could be performed on any individual subject to fewer than four. Intestinal intubation has proven to be quite effective for obtaining suitable samples of intestinal fluid for the determination of specific secretory-IgA levels (3–5, 9, 12–15, 24, 25, 36). Sampling occurred prevaccination and again either on day 14 or 15 or on day 14 or 15 and 21 following the commencement of oral vaccination, since this time point has been shown to represent the timing of the peak response following primary vaccination (12). Intestinal-fluid samples with a pH of >6.5 were collected and kept on ice until 25 ml had been collected from each subject. The samples were centrifuged at 4,000 × *g* at 4°C and stored at −70°C until required.

Saliva samples were collected from the buccal opening of one of the parotid ducts by using a Curby cap and were stored at −70°C without any further treatment (14). Saliva production was stimulated by the placement of citric acid granules on the tongue. With serial saliva samples obtained from 11 subjects on day 0 and on days 7, 14, and 21 postvaccination, it was determined that the peak salivary-IgA response occurred between days 14 and 21 in most subjects (data not shown). All serum samples were collected as detailed in Table 1 and stored as 1-ml portions at −70°C.

PBL were obtained by Ficoll-Paque (Pharmacia, Uppsala, Sweden) centrifugation of heparinized venous blood obtained on days 0 and 7 and processed as previously detailed (9).

ELISA for quantifying specific antibody. Levels of class-specific antityphoid LPS antibodies in serum and secretions were quantified by a previously described enzyme-linked immunosorbent assay (ELISA) (9). Briefly, 96-well polyvinyl microtiter ELISA plates (Costar; Data Packaging Corp., Cambridge, Mass.; catalog no. 2595) were coated with methylated bovine serum albumin (BSA)-linked *S. typhi* Ty2 LPS (9) (Sigma Chemical Co., St. Louis, Mo.; catalog no. L 6386) and blocked with 0.05% (wt/vol) BSA in phosphate-buffered saline (PBS). Subsequently, starting dilutions of serum (1:10), intestinal fluid (1:2), and saliva (undiluted) obtained from individually vaccinated subjects were added to duplicate wells on the plates and titrated twofold down the plate in a solution of 0.05% BSA in PBS. All plates were incubated at 37°C for 16 h. After washing, alkaline phosphatase-conjugated goat anti-human IgA or IgG antiserum (KPL; Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added and the plates were incubated at 37°C for 4 h. After washing, 0.100 ml of a solution (1 mg/ml) of the substrate *p*-nitrophenyl phosphate in a 10% diethanolamine buffer was added to all wells, and after further incubation at 37°C for 2 h, the plates were read by using a Titertek ELISA reader (model 310C) at 405 nm. All samples were assayed concurrently. In vitro specific antityphoid antibody production by isolated PBL was assayed as described above, except that duplicate wells of 10^6 PBL per well were incubated in RPMI 1640 cell culture medium (Flow Laboratories, Sydney, New South Wales, Australia) supplemented with 2 mmol of L-glutamine per liter in a 5% CO_2 incubator, and the substrate incubation time was 4 h at 37°C (9).

In each assay, serum obtained from a convalescent typhoid patient with known high titers of antibody directed against *S. typhi* LPS was included as a positive control and serum obtained from an unexposed individual with known low titers of antibody against *S. typhi* LPS was included as a negative control. A modified single radial immunodiffusion method was used to determine the total class-specific immunoglobulin content of intestinal fluid and saliva (13).

Specific antibody responses in serum are presented as the reciprocal of the final titration that gave an optical density of 0.15 ELISA absorbance units in 0.100 ml (the volume added to each well) and are expressed as units of antibody. These endpoints were calculated according to the method of Tijssen and represent the upper limit of the 95% confidence intervals (CI) above the mean background level (40). Specific

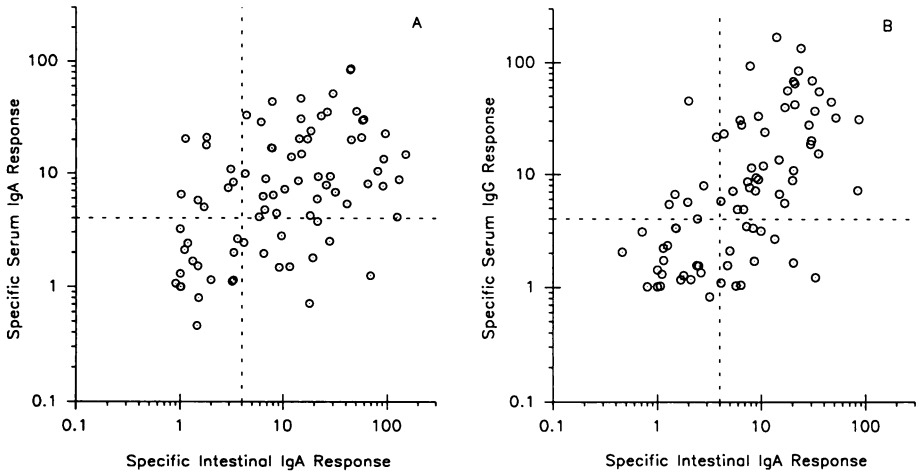

FIG. 1. The relationship between specific serum IgA and IgG and intestinal-IgA antibody responses following oral vaccination. The graph plots the magnitude of increase in specific serum IgA (A) and IgG (B) antibody titers against the adjusted postvaccination magnitudes of increase in specific antibody titers of concurrently collected samples for each individual. The dotted lines indicate fourfold increases in specific antibody following vaccination.

antibody units per 0.100 ml of intestinal fluid and saliva, obtained by ELISA as for serum, were analyzed following adjustment for total class-specific immunoglobulin content. Adjusted values were expressed as units of specific antibody per milligram of total class-specific immunoglobulin. Increases in the level of specific antibody in serum or secretions of fourfold or greater postvaccination were regarded as significant (2, 29). A PBL response was defined as an optical density of ≥0.100 ELISA absorbance units (9).

This study focused on specific IgA responses since secretory IgA is the most abundant intestinal immunoglobulin (31), specific immune responses following oral *S. typhi* Ty21a administration are most commonly in this class (9, 12, 14), and intestinal IgG and IgM concentrations are variable because of their susceptibility to proteolytic digestion despite freezing at −26°C in the presence of proteolytic inhibitors, often being undetectable following 1 month's storage (9, 35). Serum IgG responses were also included for comparison, since it has been suggested that these may be useful indicators of a local immune response (26).

For the purpose of correlating postvaccination immune responses, postvaccination increases above prevaccination titers were calculated for serum with postvaccination samples collected on day 14 or 15 and for intestinal fluid and saliva with adjusted antibody titers of a single postvaccination sample collected on day 14 or 15. The level of specific IgA antibody production in vitro by PBL was determined by using day 7 postvaccination PBL samples. All of these timings for the collection of samples have been previously determined to approximate the peak immune responses for the respective samples (3, 9, 12–14, 23).

Statistics. The significance of any differences between the postvaccination mean increases of paired sample groups was determined by Student's *t* test, with the significance level (*P* value) alone being reported. The strength of any correlations between two different measures was evaluated by using Pearson's product-moment correlation coefficient, with both *r* and *P* values being reported.

RESULTS

Antityphoid LPS intestinal-IgA response. A specific antityphoid LPS IgA antibody response of fourfold or greater was observed in 69% (56 of 81) of all subjects, with a mean increase of 35.2-fold (95% CI of 20.6 to 49.8; $P = 0.000040$). Of the 25 subjects with responses of less than fourfold, 18 had responses of less than twofold and were therefore classified as nonresponders.

Saliva as an indirect measurement of a specific intestinal-IgA response. A weak specific IgA response in the saliva of the 22 group B subjects was identified, with a mean increase of 2.34-fold (95% CI of 1.21 to 3.37). No correlation between the magnitudes of the salivary- and the intestinal-IgA specific antibody responses was found ($r = 0.0013$; $P = 0.99$). Fourfold or greater increases in postvaccination specific IgA antibody were observed in all (22 of 22) of the subjects' intestinal-fluid samples yet in only 13.6% (3 of 22) of the subjects' salivary samples. The specific salivary-IgA antibody responses were exceptionally poor in magnitude and consistency, even allowing for the small number of subjects used in the comparison.

Serum IgA and IgG as indicators of a specific intestinal-IgA response. No correlation between the increase in specific IgA responses in intestinal fluid and in serum was evident ($r = 0.18$; $P = 0.10$). From Fig. 1A, it is evident that 57% (46 of 81) of all subjects had responses of fourfold or greater in both serum and intestinal-fluid IgA, while 19.8% (16 of 81) of the subjects did not achieve such responses. Furthermore, 12.3% (10 of 81) of the subjects had an intestinal-IgA response but did not have a detectable serum response. In 11.1% (9 of 81) of the subjects in whom a serum IgA response was observed, no detectable intestinal-IgA response was evident.

As evident in Fig. 1B, a similar proportion of subjects had serum IgG responses as identified for serum IgA, with 53% (43 of 81) of the subjects having both a fourfold or greater serum IgG and intestinal-IgA response and 23.4% (19 of 81) of the subjects having no such response. As observed for serum IgA, 14.8% (12 of 81) of the subjects were noted to have intestinal-IgA responses in the absence of a serum IgG

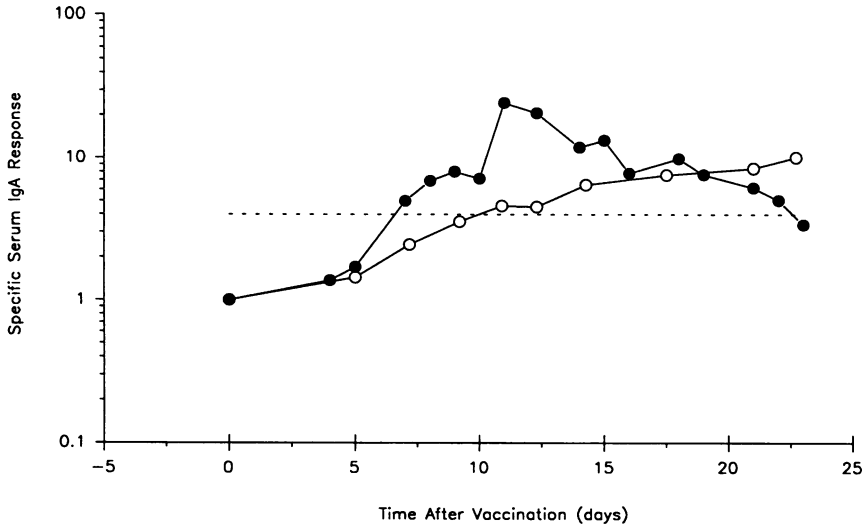

FIG. 2. Kinetics of the specific serum IgA responses in individuals orally vaccinated with live *S. typhi* Ty21a. Serum responses are given as the geometric mean magnitudes of increase in specific IgA postvaccination. The dotted lines indicate fourfold increases in specific antibody following vaccination. ●, standard response group; ○, delayed response group.

response, while 8.7% (7 of 81) of the subjects achieved serum IgG responses without a corresponding intestinal-IgA response. However, unlike the serum IgA response, a significant correlation between the postvaccination serum IgG increase and the intestinal-IgA increase was found ($r = 0.38$; $P = 0.00064$).

Effect of sampling time on the usefulness of serum IgA as an indicator of a specific intestinal-IgA response. On examination of the individual specific serum IgA results from the 36 subjects who received $\approx 10^{11}$ viable *S. typhi* Ty21a organisms and from whom serial serum samples had been obtained (groups A and B), two distinct response patterns were identified. Figure 2 depicts the geometric mean serum IgA antityphoid antibody responses as magnitudes of increase above the prevaccination titer over time for each of these two response groups. The dominant group (designated the standard response group) consisted of 83.3% (30 of 36) of these subjects and followed the kinetic pattern described previously. The serum IgA response peaked around days 11 to 12 and significantly declined, becoming an increase of less than fourfold above prevaccination levels by day 23 ($P = 0.014$).

The kinetic pattern of the specific serum IgA response of the second newly identified group (designated the delayed response group), which consisted of 16.7% (6 of 36 of the subjects, showed a slow increase in antibody response which did not peak until after day 23. The day 12 geometric mean increase for this group was significantly lower than the day 12 (peak) response of the standard response group ($P = 0.0073$). No significant difference between the groups at day 22 was observed ($P = 0.24$). This was not surprising, considering that at day 22 the standard response was declining while the delayed response was still ascending. In two delayed response subjects, for whom additional time point data were available, this response had begun to decline only at day 32 (data not shown). Furthermore, on analysis of their individual day 15 specific intestinal IgA responses, it was noticed that these six subjects either had very low responses (less than twofold) or were nonresponders. However, because of the limitations of the sampling technique, multiple intestinal fluid samplings to investigate whether the peak intestinal IgA response also occurred much later in these subjects were not possible.

Similarly, the subjects with the delayed IgA response also appeared to have a lower mean serum IgG response. However, this was not significantly different from the mean serum IgG response of the subjects with the standard IgA response because of the individual variation in the magnitude of the IgG responses that was observed.

Specific IgA production by circulating PBL as indicators of specific intestinal-IgA responses. The mean prevaccination PBL response of all 81 subjects (groups A, B, and C) was 0.019 ELISA absorbance units (95% CI of 0 to 0.041), while the mean postvaccination response was 0.867 ELISA absorbance units (95% CI of 0.716 to 1.017). Only 7.4% (6 of 81) of the subjects failed to have a specific IgA PBL response that was detectable following vaccination, and, importantly, no subject had an intestinal-IgA response in the absence of a PBL IgA response (Fig. 3A). In 25% (19 of 75) of the 75 PBL responders, a response was detectable for which no intestinal-IgA response was observed. The magnitude of the postvaccination PBL IgA response of all 81 subjects significantly correlated both with the adjusted postvaccination intestinal-IgA titer ($r = 0.44$; $P = 0.000063$) and with the postvaccination increase in intestinal-IgA titer ($r = 0.38$; $P = 0.00071$).

The PBL response also related quite well to the serum IgA response, with the magnitude of the PBL IgA response significantly correlating with that of the serum IgA response ($P = 0.42$; $P = 0.000081$). While 26% (21 of 81) of the PBL responders did not have a fourfold or greater specific serum IgA response, only one subject demonstrated a serum IgA response in the absence of a PBL IgA response (Fig. 3B).

DISCUSSION

The process of directly sampling fluid from the small intestine has been regarded as probably the optimal method for the determination of a specific immune response to an enterically delivered vaccine. As such, it could be considered the "gold standard" by which other methods may be compared. The collection of adequate samples of intestinal fluid is not always convenient or easy. Intestinal intubation

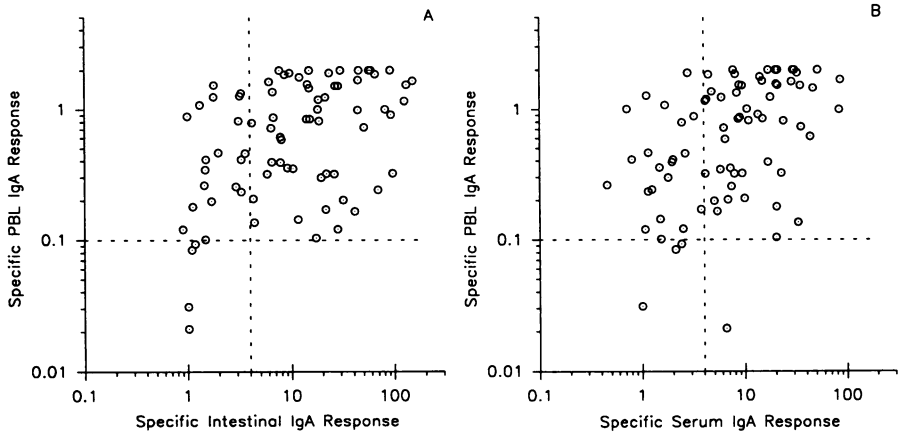

FIG. 3. The relationship between in vitro specific IgA antibody production by PBL and specific serum and intestinal-IgA responses. The graphs plot the adjusted postvaccination magnitudes of increase in specific intestinal antibody levels (A) and the magnitude of increase in specific serum IgA antibody titers (B) against the day 7 specific IgA PBL response expressed as the optical density at 405 nm per 10^6 PBL for each individual. The horizontal dotted lines indicate the optical density at 405 nm of 0.100 (previously defined as a response [9, 12]), while the vertical dotted lines indicate the fourfold increases in specific antibody following vaccination. Data from two subjects with postvaccination PBL values of 0.000 optical density units at 405 nm and serum and intestinal postvaccination increases of 1.00- and 1.00-fold, respectively, have not been included on the graph.

is a very labor-intensive, invasive, and time-consuming procedure that is not particularly pleasant for the individual undergoing it. It is also not suitable for the very young or the very old members of the community. Therefore, the need to identify alternative indicators of local intestinal immunity is crucial to the development of an enteric vaccine.

Bacterial infections of the gastrointestinal tract, as well as oral vaccines, normally produce a secretory-IgA immune response in the intestine, which may be accompanied by a more widespread response with specific secretory IgA detectable in the external secretions of other distant mucosal surfaces (1, 14, 21, 30, 33). This pattern of response has been reported to occur without the corresponding appearance of detectable specific antibodies in serum (5, 30, 35). One possible indirect indicator frequently considered has been salivary specific IgA (14, 15, 20, 21). In this study, despite substantial specific intestinal-IgA responses being evident, the specific salivary-IgA responses in samples collected concurrently with intestinal fluid were of very low magnitude, with only 3 of 22 subjects achieving responses of fourfold or more; this did not correlate with the intestinal-IgA response. The failure to adjust the salivary-IgA responses for total IgA content would have resulted in no responders being identified.

Serum antibody determination has been of limited value as a correlative indicator of an intestinal-IgA response (7, 12, 30), since significant changes in specific antibody titers are not always present following infection or vaccination (5, 21, 24). In the present study, the serum IgA and serum IgG responses were not measurable in 12.3 and 14.8%, respectively, of the subjects with specific intestinal-IgA responses. In subjects with a measurable serum response, the magnitude of the serum IgA response did not correlate with that observed in the intestine, whereas there was a significant correlation between the magnitude of the serum IgG response and the intestinal-IgA response. These findings are consistent with observations made for healthy, previously unexposed subjects (12) and with the apparent association between the serum IgG response and protection in an endemic location (26).

As noted previously for intestinal fluid (12), the timing of sample collection for serum IgA determination also appeared to be critical for the value of this determination as a useful indication of an intestinal immune response. It has been previously noted that the serum IgA peak occurs about 11 to 14 days following the commencement of oral vaccination, gradually declining in the absence of continued local stimulation to become barely significant at 6 weeks postvaccination (12). However, for the first time, the present study identified two distinct patterns of serum IgA immune response, which were labelled standard and delayed response patterns. The standard response pattern represented the serial serum IgA response that has been described previously (9, 12, 14). There was a rapid increase in specific serum IgA, which peaked around days 11 to 14 following the first dose and declined during the next 2 to 3 weeks. The delayed response pattern, which accounted for 16.6% of the subjects for whom serial samples were available, did not peak until much later. This study was not primarily designed to identify this group; therefore, at the end of serum collections, the specific IgA antibody titer in this group was still increasing. There were no apparent differences in vaccine doses or formulations or previous *Salmonella* experiences between these subjects and those following the standard response pattern that could have contributed to these response differences. Since *Salmonella* infections may to a degree be under genetic control (34), it is possible that a delayed IgA response pattern reflects a genetic variation in the response to *S. typhi* rather than a generalized phenomenon. This may also account for the failure to achieve responses of fourfold or greater from the day 14 or 15 intestinal-fluid samples of any of these subjects. While these findings require further investigation, it is important to note that had only a single day 12 sampling time point been chosen to assess the immune responsiveness of these particular individuals, most results for the delayed-response-group subjects would have been recorded as vaccine failures.

The observation of two apparently distinct groups of IgA responders may have contributed to the failure of serum IgA responses to correlate. The serum IgG response appeared

consistent even with the delayed IgA responders, following a response pattern similar to that observed for the serum IgG of the standard IgA responders. Although these were of a lower magnitude at day 14, they were not significantly lower.

In 11.1% (9 of 81) and 8.6% (7 of 81) of the subjects who had significant serum IgA and/or IgG responses, a correspondingly significant intestinal-IgA antibody response was not detectable. This observation casts doubt on the reliability of intestinal-IgA determination as the gold standard. From this work, it would appear that a single measurement that uses either serum or a secretion would be unlikely to be sufficiently reliable to determine whether a subject has mounted a specific immune response to an orally administered bacterial vaccine and that both serum and intestinal-IgA antibody levels need to be measured together.

Of all the indirect measurements considered in this paper, the one most likely to provide the most accurate indicator of a local immune response appeared to be the measurement of day 7 circulating PBL IgA specific antibody response. Determination of specific antibody production by PBL that circulate following mucosal exposure to an antigen has been demonstrated to be a useful measurement of local mucosal immunity (8, 9, 12, 22, 23). However, direct comparisons between the responses observed by using these assays and how the assays relate to the responses, especially the magnitudes of the responses observed in the intestine, are lacking. An earlier study that used a dozen subjects vaccinated with *S. typhi* Ty21a demonstrated a strong association between local intestinal specific antibody and the peak antibody response in subjects' circulating PBL (9). The much larger comparison reported here identified considerably more responders following vaccination and was able to directly demonstrate highly significant correlations between the day 7 postvaccination specific IgA PBL response in vitro and both the day 12 or 14 postvaccination intestinal-IgA titer and the magnitude of the postvaccination increase in intestinal-fluid IgA. If we continue to assume that intestinal IgA is the gold standard, then many of these PBL responses may represent false-positive results. However, with the evidence from the serum IgA and IgG comparisons, it is probably safe to propose that all of the PBL responses observed here were real responders and that the intestinal-IgA measurement lacked sensitivity.

Since neither intestinal-fluid nor serum IgA or IgG determinations alone can confidently identify all patients who have generated a primary local intestinal immune response to an orally administered vaccine, the PBL specific antibody response alone may provide a sensitive, correlative measurement of such an immune response which can be easily performed and which may be suitable for community studies. However, it is possible that the assay of PBL specific-antibody production described here detects nonrelevant immune responses, and, to date, its value as a predictive measure of protection against virulent challenge has not been adequately evaluated.

ACKNOWLEDGMENTS

I thank J. T. LaBrooy, D. J. C. Shearman, and the staff of the Department of Medicine at the Royal Adelaide Hospital, Adelaide, Australia, without whose support these studies could not have been performed.

The vaccine clinical studies referred to were supported by Enterovax Limited, Salisbury, Australia.

REFERENCES

1. **Allardyce, R. A., D. J. C. Shearman, D. B. L. McClelland, K. Marwick, A. J. Simpson, and R. B. Laidlaw.** 1974. Appearance of specific colostrum antibodies after clinical infection with *Salmonella typhimurium*. Br. Med. J. **3**:307–309.
2. **Attridge, S. R., C. Dearlove, L. Beyer, L. van den Bosch, A. Howles, J. Hackett, R. Morona, J. LaBrooy, and D. Rowley.** 1991. Characterization and immunogenicity of EX880, a *Salmonella typhi* Ty21a-based clone which produces *Vibrio cholerae* O antigen. Infect. Immun. **59**:2279–2284.
3. **Bartholomeusz, R. C. A., B. D. Forrest, J. T. LaBrooy, P. L. Ey, D. Pyle, D. J. C. Shearman, and D. Rowley.** 1990. The serum polymeric IgA antibody response to typhoid vaccination: its relationship to the intestinal IgA response. Immunology **69**:190–194.
4. **Bartholomeusz, R. C. A., J. T. LaBrooy, M. Johnson, D. J. C. Shearman, and D. Rowley.** 1986. Gut immunity to typhoid: the immune response to a live oral typhoid vaccine Ty21a. J. Gastroenterol. Hepatol. **1**:61–67.
5. **Chau, P. Y., R. S. W. Tsang, S. K. Lam, J. T. LaBrooy, and D. Rowley.** 1981. Antibody response to the lipopolysaccharide and protein antigens of *Salmonella typhi* during typhoid infection. II. Measurement of intestinal antibodies by radioimmunoassay. Clin. Exp. Immunol. **46**:515–520.
6. **Clemens, J. D., M. Jertborn, D. Sack, B. Stanton, J. Holmgren, M. R. Khan, and S. Huda.** 1986. Effect of neutralization of gastric acid on immune responses to an oral B subunit, killed whole-cell cholera vaccine. J. Infect. Dis. **154**:175–178.
7. **Clemens, J. D., F. van Loon, D. A. Sack, J. Chakraborty, M. R. Rao, F. Ahmed, J. R. Harris, M. R. Khan, M. Yunus, S. Huda, B. A. Kay, A.-M. Svennerholm, and J. Holmgren.** 1991. Field trial of oral cholera vaccines in Bangladesh: serum vibriocidal and antitoxic antibodies as markers of the risk of cholera. J. Infect. Dis. **163**:1235–1242.
8. **Czerkinsky, C., S. J. Prince, S. M. Michalek, S. Jackson, M. W. Russell, Z. Moldoveanu, J. R. McGhee, and J. Mestecky.** 1987. IgA antibody-producing cells in peripheral blood after antigen ingestion: evidence for a common mucosal immune system. Proc. Natl. Acad. Sci. USA **84**:2449–2453.
9. **Forrest, B. D.** 1988. The identification of an intestinal immune response using peripheral blood lymphocytes. Lancet **i**:81–83.
10. **Forrest, B. D.** 1988. The development of a bivalent vaccine against diarrhoeal disease. Southeast Asian J. Trop. Med. Public Health **19**:449–457.
11. **Forrest, B. D., J. T. LaBrooy, S. R. Attridge, G. Boehm, L. Beyer, R. Morona, D. J. C. Shearman, and D. Rowley.** 1989. Immunogenicity of a candidate live oral typhoid/cholera hybrid vaccine in humans. J. Infect. Dis. **159**:145–146.
12. **Forrest, B. D., J. T. LaBrooy, L. Beyer, C. E. Dearlove, and D. J. C. Shearman.** 1991. The human humoral immune response to *Salmonella typhi* Ty21a. J. Infect. Dis. **163**:336–345.
13. **Forrest, B. D., J. T. LaBrooy, C. E. Dearlove, and D. J. C. Shearman.** 1992. Effect of parenteral immunization on the intestinal immune response to *Salmonella typhi* Ty21a. Infect. Immun. **60**:465–471.
14. **Forrest, B. D., J. T. LaBrooy, P. Robinson, C. E. Dearlove, and D. J. C. Shearman.** 1991. Specific immune response in the human respiratory tract following oral immunization with live typhoid vaccine. Infect. Immun. **59**:1206–1209.
15. **Forrest, B. D., D. J. C. Shearman, and J. T. LaBrooy.** 1990. Specific immune response in humans following rectal delivery of live typhoid vaccine. Vaccine **7**:209–212.
16. **Germanier, R., and E. Furer.** 1975. Isolation and characterization of galE mutant Ty 21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. J. Infect. Dis. **131**:553–558.
17. **Giannella, R. A., S. A. Broitman, and N. Zamcheck.** 1973. Influence of gastric acidity on bacterial and parasitic infections. Ann. Intern. Med. **78**:271–276.
18. **Gilman, R. H., R. B. Hornick, W. E. Woodward, H. L. DuPont, M. J. Snyder, M. M. Levine, and J. P. Libonati.** 1977. Evaluation of a UDP-glucose-4-epimerase-less mutant of *Salmonella typhi* as a live oral vaccine. J. Infect. Dis. **136**:717–723.
19. **Herrington, D. A., L. van den Verg, S. B. Formal, T. L. Hale, B. D. Tall, S. J. Cryz, E. C. Tramont, and M. M. Levine.** 1990. Studies in volunteers to evaluate candidate *Shigella* vaccines:

further experience with a bivalent *Salmonella typhi-Shigella sonnei* vaccine and protection conferred by previous *Shigella sonnei* disease. Vaccine **8:**353–357.

20. **Jertborn, M., A.-M. Svennerholm, and J. Holmgren.** 1984. Gut mucosal, salivary and serum antitoxic and antibacterial antibody responses in Swedes after oral immunization with B subunit-whole cell cholera vaccine. Int. Arch. Allergy Appl. Immunol. **75:**38–43.
21. **Jertborn, M., A.-M. Svennerholm, and J. Holmgren.** 1986. Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. J. Clin. Microbiol. **24:**203–209.
22. **Kantele, A.** 1990. Antibody-secreting cells in the evaluation of the immunogenicity of an oral vaccine. Vaccine **8:**321–326.
23. **Kantele, A., H. Arvilommi, and I. Jokinen.** 1986. Specific immunoglobulin-secreting human blood cells after peroral vaccination against *Salmonella typhi*. J. Infect. Dis. **153:**1126–1131.
24. **LaBrooy, J. T., G. P. Davidson, D. J. C. Shearman, and D. Rowley.** 1980. The antibody response to bacterial gastroenteritis in serum and secretions. Clin. Exp. Immunol. **41:**290–296.
25. **LaBrooy, J. T., D. J. C. Shearman, and D. Rowley.** 1982. Antibodies in serum and secretions 1 year after salmonella gastroenteritis. Clin. Exp. Immunol. **48:**551–554.
26. **Levine, M. M., C. Ferreccio, R. E. Black, C. O. Tacket, R. Germanier, and the Chilean Typhoid Committee.** 1989. Progress in vaccines against typhoid fever. Rev. Infect. Dis. **11**(Suppl. 3)**:**S552–S567.
27. **Levine, M. M., D. Herrington, J. R. Murphy, J. G. Morris, G. Losonsky, B. Tall, A. A. Lindberg, S. Svenson, S. Baqar, M. F. Edwards, and B. Stocker.** 1987. Safety, infectivity, immunogenicity, and *in vivo* stability of two attenuated auxotrophic mutant strains of *Salmonella typhi*, 541Ty, and 543Ty, as live oral vaccines in humans. J. Clin. Invest. **79:**888–902.
28. **Levine, M. M., J. B. Kaper, D. Herrington, J. Ketley, G. Losonsky, C. O. Tacket, B. Tall, and S. Cryz.** 1988. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103Hg-R. Lancet **ii:**467–470.
29. **Levine, M. M., J. B. Kaper, D. Herrington, G. Losonsky, J. G. Morris, M. L. Clements, R. E. Black, B. Tall, and R. Hall.** 1988. Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. Infect. Immun. **56:**161–167.
30. **Mestecky, J., J. R. McGhee, R. R. Arnold, S. M. Michalek, S. J. Prince, and J. L. Babb.** 1978. Selective induction of an immune response in human external secretions by ingestion of bacterial antigen. J. Clin. Invest. **61:**731–737.
31. **Mestecky, J., M. W. Russell, S. Jackson, and T. A. Brown.** 1986. The human IgA system: a reassessment. Clin. Immunol. Immunopathol. **40:**105–114.
32. **Migasena, S., P. Pitisuttitham, B. Prayurahong, P. Suntharasamai, W. Supanaranond, V. Desakorn, U. Vongsthongsri, B. Tall, J. Ketley, G. Losonsky, S. Cryz, J. B. Kaper, and M. M. Levine.** 1989. Preliminary assessment of the safety and immunogenicity of live oral cholera vaccine strain CVD 103Hg-R in healthy Thai adults. Infect. Immun. **57:**3261–3264.
33. **Nayak, N., N. K. Ganguly, B. N. S. Walia, V. Wahi, S. S. Kanwar, and R. C. Mahajan.** 1987. Specific secretory IgA in the milk of *Giardia lamblia*-infected and uninfected women. J. Infect. Dis. **155:**724–727.
34. **Naylor, G. R. E.** 1983. Incubation period and other features of food-borne and water-borne outbreaks of typhoid fever in relation to pathogenesis and genetics of resistance. Lancet **i:**864–866.
35. **Panosian, C. B., and L. S. Gorbach.** 1985. Infectious diseases of the gastrointestinal tract, p. 165–187. *In* J. I. Gallin and A. S. Fauci (ed.), Mucosal immunity. Raven Press, New York.
36. **Samson, R. R., D. B. L. McClelland, and D. J. C. Shearman.** 1973. Studies on the quantitation of immunoglobulin in human intestinal secretions. Gut **14:**616–626.
37. **Svennerholm, A.-M., L. Gothefors, D. A. Sack, P. K. Bardhan, and J. Holmgren.** 1984. Local and systemic antibody responses and immunological memory in humans after immunization with cholera B subunit by different routes. Bull. W.H.O. **62:**909–918.
38. **Svennerholm, A.-M., J. Holmgren, D. A. Sack, and P. K. Bardhan.** 1982. Intestinal antibody responses after immunisation with cholera B subunit. Lancet **i:**305–308.
39. **Tacket, C. O., B. Forrest, R. Morona, S. R. Attridge, J. T. LaBrooy, B. D. Tall, M. Reymann, D. Rowley, and M. M. Levine.** 1990. Safety, immunogenicity, and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from *Salmonella typhi* Ty21a. Infect. Immun. **58:**1620–1627.
40. **Tijssen, P.** 1985. Practice and theory of enzyme immunoassays. Elsevier Science Publishers, Amsterdam.
41. **World Health Organization.** 1979. Intestinal immunity and vaccine development: a WHO memorandum. Bull. W.H.O. **57:**719–734.
42. **World Health Organization.** 1982. Oral enteric vaccines. EURO reports and studies no. 63. World Health Organization, Copenhagen.