ELSEVIER

Advanced Drug Delivery Reviews 18 (1995) 53–71

advanced
drug delivery
reviews

Clinical investigation of gut immune responses

Anne Ferguson[a,*], Jamal Sallam[a], Seamus O'Mahony[a], Ian Poxton[b]

[a]*University of Edinburgh Department of Medicine, Western General Hospital, Edinburgh EH4 2XU, UK*
[b]*Department of Medical Microbiology, Western General Hospital, Edinburgh EH4 2XU, UK*

Accepted June 1995

Abstract

Mucosal and systemic immune systems are quite separate in their properties and in the factors which regulate them. In man, assessment of immune status at gut level, including the capacity to respond to oral vaccines, should follow the general lines which are used in research and clinical investigation of systemic immunity. All of the currently available methods for direct studies of gut immunity have limitations, but a new technique, per-oral whole gut lavage with non-absorbable cleansing fluid, provides a system of gut perfusion. The value of this technique is illustrated by data on intestinal antibodies to bacterial LPS core antigens, of gut immunity in healthy African children, on the isotype of antibodies elicited by oral killed whole cell/cholera toxin B vaccine in coeliac disease patients, and on the effects of smoking on intestinal antibodies elicited by the oral typhoid vacine Ty21a in healthy UK adults. Finally, results of a recent comprehensive study show that immunological tests using faeces are likely to be highly misleading.

Keywords: Intestinal antibody; Intestinal lymphocyte; Copro-antibody; Faecal immunology; Cholera toxin; Oral cholera vaccine; Oral typhoid vaccine

Contents

* Corresponding author: Fax 0131-537-1007.

SSDI 0169-409X(95)00050-X

1. Introduction

The functions of the gastrointestinal tract include the secretion of fluids and digestive enzymes; the absorption of nutrients; mixing and propulsion of foods, digestive secretions and the gut microflora; and the neuro-hormonal coordination of these phenomena. These digestive processes have profound effects on the quantities and molecular forms of antigens within the gut lumen and tissues, and thus also on local immune reactions. Gastro-intestinal infections may directly damage the tissues or cells, or may only affect functions such as secretion or motility. Local protective immunity will influence the severity of these pathological effects on the intestines in addition to direct effects on the viability and pathogenicity of the organisms concerned. Perhaps because of the quite different properties of secretory IgA when compared with serum IgG antibodies, it appears that a vigorous local immune response does not necessarily confer protection against re-infection, or even lead to complete elimination of an intestinal pathogen, although local immunity probably influences symptom severity.

The several types of immune responses in the gut are, normally, strictly regulated. Such active immune responses may be host-protective, e.g. conferring resistance to bacterial infection, neu-

tralising viruses or toxins; the immune responses may occur but may be completely irrelevant to any protective or other function of the gut (as in most reactions between antibodies and food antigens); and from time to time the immune responses cause tissue damage, for example by delayed type hypersensitivity (DTH) reactions, IgG- or IgM-mediated complement-fixing hypersensitivity, or IgE-mediated local anaphylaxis. Hypersensitivity may be entirely inappropriate, and thus a primary cause of disease (e.g. to gluten in coeliac disease), but also may be an unavoidable side effect of a protective immune response, as is well recognised in tuberculosis and leprosy, and in the elimination phase of helminth parasite expulsion from the gut.

Much research on gut immunology has been conducted in mice. It is likely that the general principles derived from the results of such experimental work are also applicable in man, but this has been proven for only a few aspects of immunity. Good clinical studies of the physiology and regulation of the human GALT are urgently required.

2. Mucosal immunity is distinct from systemic immunity

The science of mucosal immunology has developed rapidly during the last 20 years, and many of the principles and concepts are different from those of classical systemic immunity. A recently published handbook [1] gives a comprehensive account of the biology of the gut epithelium, mucosal defence mechanisms, mucosal immunoglobulins, lymphoid cells, regulation of induction and expression of immunity, inflammation, immunisation and vaccines, and is highly recommended as a source of background information to this paper.

2.1. Patterns of immune responses to fed antigen

The immune system of the gut has a number of different roles. When the route of entry is through follicle-associated epithelium of Peyer's patches of immunologically normal and mature mammals, the general trend is towards suppression of immunity–in other words, *oral tolerance.* However, active immunisation may also follow the feeding of antigen and this is typically in the form of harmless *secretory IgA antibody.* In some circumstances there is, however, induction of potentially immunopathogenic immune reactions when antigen is fed, for example *IgE, IgG antibody or T cell mediated immunity.* Thus there may be either induction or suppression of a particular immune response, that is antibody- or T cell-mediated. Regulation of immunity is critical to normal gut function, and, of course, vaccine design and delivery must lead to active immunity without hypersensitivity, but not oral tolerance.

2.2. Induction and expression of immunity

Overall, mucosal immune functions can be separated into induction and effector phases.

The *induction* of a specific immune response is critically dependent on the situation of the individual when antigen is first encountered. Factors such as age, dose, route of encounter and physico-chemical form of antigen critically influence the type of immune response thatwill predominate for the rest of the life of the individual. The state of activation of antigen-processing cells and T cells in the tissues where antigen is first encountered are also important factors.

In an immune individual, re-exposure to antigen will result in *expression* of an antibody-mediated or cell-mediated specific immune response, either at the site of exposure or, if the antigen is absorbed or otherwise transferred around the body, at other sites or systemically. As discussed below, there are circumstances when a local immune response in the gut causes disease such as malabsorption. A key factor in such immune-mediated tissue damage is the capacity of the tissue to respond to stimuli such as cytokines. The impaired capacity to mount non-specific inflammation, such as occurs with some nutrient deficiencies, may in fact protect the gut epithelium from some of the potentially adverse effects of a mucosal DTH reaction. We have observed failure of the usual crypt hy-

perplasia associated with intestinal DTH in protein malnourished mice [2].

2.3. Immunoregulation

There are many T lymphocytes dispersed in the mucosa as well as in the organised lymphoid tissues of the gut. T cells are critical to the induction of appropriate immune responses when antigen is encountered via the gut lumen, and also as important effector cells for local protective and hypersensitivity reactions.

Handling of antigen by the gut is also important. Experiments with protein antigens have shown that there is subtle alteration – "processing" of antigen as it crosses the gut epithelium – and such material is tolerogenic for cell-mediated immunity, rather than immunogenic [3–5]. In all normal mammals antigenic molecules gain access from the gut lumen into the tissues of the body, but immune responses to such materials do not normally cause disease.

2.4. Spectrum of immune reactions to cholera toxin in mice

Experiments which we conducted in mice, using cholera toxin (CT) as a fed antigen, illustrate very well the distinct differences between the mucosal and systemic compartments, humoral and cellular immunity, active immunisation and tolerance [6–8]. Cholera toxin is a most unusual antigen with considerable adjuvant activity when given orally and we found, as did several other investigators, that a single oral dose of CT induced not only intestinal but also a serum antibody response. However, the cellular limb of immunity (assessed by a footpad swelling test of DTH after later, systemic immunisation) was down-regulated; in other words, oral tolerance for DTH was induced at the same time as active humoral immunisation. Results of a typical experiment [7] are shown in Fig. 1. The legend gives details of the experimental protocol and an interpretation of the results. Tolerance was antigen-specific, dose-dependent and transferable by spleen suppressor lymphocytes. Oral tolerance for DTH could also be transferred by serum obtained from mice fed CT 1 h previously; thus, CT is 'gut-processed' to a tolerogenic form during absorption.

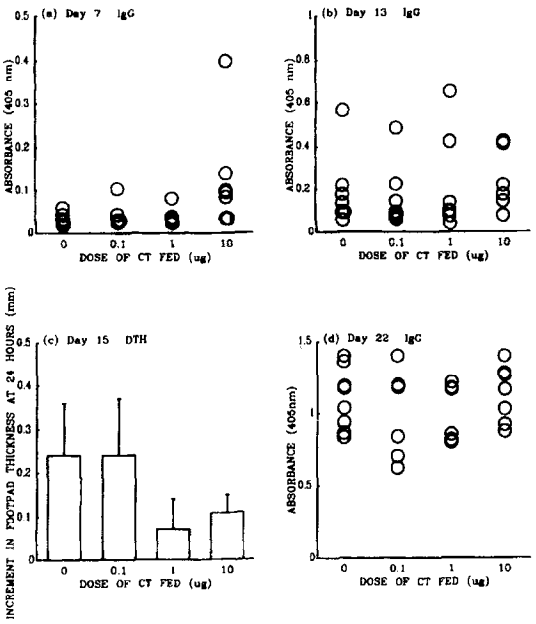

Fig. 1. The effect of prefeeding different doses of CT. Animals were fed either ABS (inert buffer) or 0.1, 1 or 10 μg CT (day −7) 1 week before immunization with 1 μg CT in complete Freund's adjuvant (day 0). 2 weeks later (day 14), they were challenged with 5 μg toxoid into a footpad and their DTH responses measured as footpad swelling after 24 h. Animals were bled on days 7, 13 and 22 of the experiment for assessment of their serum antitoxin antibody responses. The IgG antitoxin responses of animals fed either 0.1 or 1 μg CT were no different from ABS-fed controls at any time. However, animals fed 10 μg CT had significantly higher IgG antitoxin responses ($p < 0.02$) on day 7. In contrast, animals fed either 1 or 10 μg CT had suppressed DTH responses ($p < 0.001$ and $p < 0.01$, respectively) but a dose of 0.1 μg CT did not significantly inhibit the induction of DTH. The graphs show the IgG antitoxin antibody response in serum on days 7 (a), 13 (b) and 22 (d) and the DTH response on day 15 (c).

3. Required information for full clinical description of mucosal immunity

Assessment of immune status at gut level, including the capacity to respond to oral vaccines, can follow the general lines which have proved invaluable in research and clinical investigation of systemic immunity.

3.1. Structured evaluation of systemic immune status

Protocols for the clinical evaluation of systemic immunity which combine general clinical features, in vivo and in vitro laboratory tests, are agreed and widely applied by clinical and laboratory immunologists, and are used in the the investigation and management of patients with primary, acquired and iatrogenic immunodeficiency syndromes. Aberrant immunity can either be perceived and classified in relation to whether the problem lies in primary lymphoid organs, the peripheral lymphoid system, or in the non-antigen specific cellular or humoral mechanisms. A different approach, perhaps of greater relevance for day to day therapeutic decisions, classifies the patient by the type of effector mechanism involved, for example T cell mediated immunity, immunoglobulin isotype, polymorphonuclear or mononuclear cell function.

3.1.1. Clinical history

Important features to be elicited include family history, of immunodeficiency states, autoimmune disease and atopy. Evidence of previous normal recovery from bacterial or viral infections is important. Response to immunisation, particularly with live vaccines (smallpox, BCG) can give valuable evidence of normal cell mediated immunity. The aberrant immune status of atopy can usually be implied from the history.

3.1.2. Cells and cell-mediated immunity

There are now a range of techniques for defining lymphocyte subsets in blood, extending into research orientated observations. Evidence of the existence of specific cell mediated immunity (implying both normal afferent and efferent limbs) is obtained by in vivo tests of delayed type hypersensitivity using a range of recall antigens such as tuberculin, and many in vitro tests of antigen reactive T cell function are available, ranging from antigen driven blast transformation to the secretion of cytokines in culture with antigen. Usually, these tests are done with peripheral blood lymphocytes.

3.1.3. Immunoglobulins and antibodies

Almost invariably, assays of total immunoglobulins and of subclasses are done on serum, by a variety of techniques in which monoclonal or polyclonal anti-heavy chain antisera are used.

Apart from the use of immediate skin tests for in vivo detection of IgE class antibodies, tests for the presence, titres, avidity etc of antibody are also studied using serum. Precise information on induction and expression of humoral immunity is obtained by studying the primary and secondary immune responses to defined antigens not previously encountered, for example, key-hole limpet haemocyanin.

3.1.4. Other immune parameters

A wide range of other cellular and humoral components may be aberrant and a full immune status evaluation should include an appraisal of eosinophils, mast cells, basophils, complement, and reticuloendothelial function.

3.2. Evaluation of gastrointestinal immune status (Table 1)

3.2.1. Antigen handling

The afferent limb, involving the follicle associated epithelium and organised lymphoid tissues such as Peyer's patches, tonsils, appendix, is rarely investigated in man. Operative specimens and endoscopically targetted biopsies of ileal Peyer's patches will provide tissues for such studies. Operative and post mortem specimens of mesenteric lymph nodes can also be examined.

3.2.2. Cells

Most workers have concentrated on cells serving effector functions, and ideally, a range of properties should be assessed.

Mucosal biopsies can safely be taken from most levels of the gastrointestinal tract, and by using micro techniques and multiple biopsies, both histological and in vitro methodscan be applied. Immunofluorescence or immunoenzyme stains, using polyclonal or monoclonal antibodies targetted to either membrane or cytoplasmic antigens, have greatly expanded the information which can be accrued from mucosal biopsy histopathology.

Table 1
Studies required for comprehensive investigation of effector immune mechanisms in the human gut

1. Specific IgA antibodies
2. Potentially immunopathogenic but also potentially useful IgM, IgG, IgE antibodies
3. Antigen-specific T cells (currently the only method available is based on intestinal antigen challenge, multiple biopsies and morphometric analysis of these biopsies)
4. T cell and macrophage activation by immunological marker studies or measurement of activation products
5. Polymorphonuclear activation
6. Immunoregulatory molecular signals
7. Inflammatory cytokines
8. Other molecular mediators of inflammation

In order to study the function of mucosal lymphocytes in vitro, it is necessary to prepare cell suspensions and many techniques have now been described and applied to operative and per-endoscopic biopsies. It is advisable to separate the intraepithelial lymphoid cells from the lamina propria cells since histological studies have shown that these are two quite separate microenvironments. In general, methods available concern non-antigen specific functions such as helper activity, suppressor inducer, suppressor cytotoxic and natural killer cell functions.

3.2.3. Specific cell-mediated immunity

Currently there are no standard methods for detecting the presence of antigen-specific T effector cells in the mucosae.

The existence of an on-going delayed-type hypersensitivity (DTH) reaction in the small intestinal and/or the colonic mucosa can be inferred by a cluster of features defined on the basis of work on experimental animals [9]. These include villus atrophy, crypt hyperplasia, and a high intra-epithelial lymphocyte count. Currently, it is only by manipulation of antigen encounter, usually of dietary protein antigens such as gluten or milk, and by repeat biopsies of the small intestinal mucosa, that the diagnosis of food protein sensitive enteropathy is made. The recovery and reappearance of the cluster of features in keeping with DTH tend to suggest that a cell mediated immune reaction is taking place in the mucosa, but this is as yet very indirect evidence.

It seems likely that mucosal cellular immunity is critical to protection from many gut pathogens, and work in man on induction of specific T cell mediated immunity to vaccine components is greatly hindered by the absence of clinical study methods.

3.2.4. Immunoglobulins and antibodies

Immunoglobulins and antibodies have been more extensively studied, and methods available for use in the study of mucosal vaccine responses are discussed in full below. Antigens which are used to evaluate intestinal secretory antibodies include those of gut micro-organisms, vaccines and dietary proteins. Just as with the systemic immune apparatus, the generation of a primary antibody response to a new antigen should be incorporated in any full protocol of evaluation of immune status.

3.2.5. Regulation of induction of active immunity and tolerance

As has been mentioned above, the usual systemic immune response when antigen is first encountered via the gut is down-regulation of subsequent immune capacity. Oral tolerance can be easily evaluated in groups of inbred animals with several necessary controls included [3–5]. Considerable ingenuity is required to study this

phenomenon in man but it is possible, for example by using keyhole limpet haemocyanin [10].

Experience with immunity to foods indicates that intestinal antigen-specific T cell mediated immunity does not normally develop to enterically encountered antigens. Whether this is true tolerance, i.e. antigen-specific suppression, or whether it is merely the absence of this limb of the effector immune response at gut level, remains to be ascertained.

4. Source materials for humoral immunity studies

Animal and clinical studies show that with very few exceptions, tests on blood antibodies and circulating cells and cytokines (components of the systemic immune system), are virtually useless as indices of mucosal immunity at gut level. Some general information on the function of the mucosa-associated lymphoid tissues can be obtained from studies of saliva or tears but these materials cannot provide organ-specific information relevant to the gut.

Unfortunately, for direct studies of gut immunity, all of the currently available methods have limitations. Considerable information can be obtained by histological and other procedures applied to small bowel or colonic mucosal biopsies, and fluid for immunological investigations may be aspirated from the jejunum or colon. How closely results obtained with these samples reflect events in other parts of the GI tract is uncertain. Furthermore, though there are many circumstances in which it is reasonable, and ethical, to intubate or biopsy symptomatic adults or children for research purposes, parallel data from clinically unaffected age-matched population controls are not easily available, and proper interpretation of the results obtained with the patients may thus be impossible.

4.1. Mucosal biopsies

It is possible to detect and count antibody-containing plasma cells in tissue sections by using isotope-labelled antigen and autoradiography, or by immunofluorescence or immunoenzyme techniques. With double-labelling methods, the isotype of antibody can also be determined. These approaches were extensively used in critical early research on mucosal immumity, using cholera toxin and tetanus toxin, in addition to soluble protein antigens such as hen egg ovalbumin.

Single cell suspensions of lamina propria cells, prepared by digestion and centrifugation of macerated biopsies, are suitable for in vitro-tests of antibody production such as the ELISPOT [13]. Antibody-producing cells can also be detected by combining culture and ELISA-based antibody detection [14].

4.2. Directly aspirated intestinal secretions

Direct jejunal intubation allows aspiration of upper small intestinal fluid [15], and similarly, duodenal or colonic fluid can be aspirated during upper GI endoscopy or colonoscopy [16], or through the tubing of a small bowel biopsy capsule [17]. When treated with protease inhibitors and with appropriate storage conditions, these may yield much useful information on antibody production in these localised segments of the gut.

4.3. Segmental perfusion

Perfusion of a defined segment of the small intestine [18] or recto-sigmoid [19], with occluding balloons above and below, allows measurements of locally-produced fluid, electrolytes, immunoglobulins, cytokines, plasma-derived proteins and other materials relevant to gut immunity. Basal and stimulated states can be assessed. This powerful approach has not, to our knowledge, been used to study vaccine responsiveness in man.

4.4. Whole gut lavage

Peroral gastrointestinal lavage with non-absorbable fluid is now extensively used to cleanse the gastrointestinal tract prior to colonoscopy, barium enema examination or colonic surgery. This lavage fluid contains abundant quantities of IgA [20], and, in disease, also IgM and IgG [21]. After filtration and processing with protease

inhibitors, ELISA techniques can readily be used to study immunoglobulins and specific antibodies, as discussed below.

4.5. Critical importance of specimen handling and storage

Human intestinal secretions, including jejunal fluid and whole gut lavage fluid (WGLF) contain many and abundant proteases. If protease inhibitors are not added to these materials immediately after collection there is rapid degradation of proteins, including antibodies [20]. For example, we found in our early studies of WGLF that delays of 1 or 2 h in the addition of protease inhibitors resulted in loss of up to 92% of the IgA [21]. We also noted that early faecally-contaminated specimens had much lower concentrations of IgA than later clear specimens. Once WGLF specimens were clear, there was no significant difference in IgA content between serial specimens. In careful experiments with jejunal fluid, Forrest made the important observation that heat-inactivation of proteases was an unsatisfactory method, leading to considerable losses of IgA and antibodies [22]; he also suggested that data reported should take into account the total concentration of IgA in the fluid being examined.

5. Validation and applications of the whole gut lavage technique

Gut lavage with large volumes of saline has been used for some time to study intestinal antibodies in the clear fluid passed per rectum after ingestion of 10–20 l of the fluid [23]. In 1988, Gaspari et al. [20] described a more acceptable technique, using 3–4 l of a non-absorbable, commercially available polyethylene glycol (PEG)-based bowel cleansing fluid. We followed up this approach [21] and have since found that in patients with intestinal inflammatory diseases, this whole gut lavage fluid (WGLF) contains immunoglobulins, antibodies, plasma-derived proteins, inflammatory cells and cytokines [17,24–34]. We have recently completed a study of fluid intake/output data, and concentrations of various substances in sequentially passed specimens, confirming that WGLF is a gut perfusate [35].

5.1. Clinical and laboratory protocols and methods

5.1.1. Clinical procedure

The lavage solution (available commercially as Klean-prep (Norgine, Oxford, UK)) contains in one sachet: 59 g PEG with a molecular weight of 3350; 1.45 g NaCl; 1.63 g $NaHCO_3$; 5.68 g Na_2SO_4; and 0.75 g KCl (BP). One sachet is dissolved in 1 l of tap water to give an osmolality of 260 mosm/l.

After an overnight fast, supervised and monitored by an experienced research nurse, patients or healthy volunteers drink the lavage solution, aiming for a rate of 200 ml every 12 min. After a period ranging from 30 min to 3.5 h, several formed or semi-liquid stools are passed, followed by faecal-stained fluid. These are discarded until clear fluid, resembling urine, is being passed per rectum. There is a very high success rate when the procedure is conducted as described–more than 95% in a series of hospital in-patients ranging in age from 14 to 89 years [36].

5.1.2. Processing of whole gut lavage fluid [26]

20 ml of clear fluid are filtered through GF/A (Whatman) glass fibre filters. To 10 ml of the filtered fluid the following reagents are added, with mixing after each addition (final concentrations in parentheses): soya bean trypsin inhibitor in phosphate-buffered saline (PBS) (80 μg/ml); sodium ethylenediaminetetracetate in PBS (15 mM); phenylmethylsulphonyl fluoride in 95% ethanol (2 mM); sodium azide (1 mM); newborn calf serum (5% v/v).

Aliquots of processed WGLF are stored at −70°C for later analyses.

5.1.3. Assay techniques

Details are published elsewhere of the methods used for assays of immunoglobulin concentrations [17], isotype-specific antibodies to dietary protein [17] and bacterial [31–33] antigens, haemoglobin [28], plasma-derived proteins [26], cytokines [29], and for cytological examination [30] of WGLF.

5.2. Validation as a whole gut perfusion

Eight patients participated in this study after giving written informed consent [35]. The standard method described above was used, but they continued to drink the lavage solution until several clear 'stools' had been passed. The time at which each specimen was passed, and its volume, were recorded. Rates of ingestion ranged between 0.8 and 1.2 l per h; the time from the start of the procedure until clear fluid was being passed varied from 40 min (in an ileostomy patient) to 5.25 h (in a very constipated patient) but, in all eight cases, rate of output significantly paralleled intake. Concentrations of various substances assayed (polyethylene glycol, residual protease activity, total proteins, IgA, IgM, IgG, albumin, α_1-antitrypsin and titres of IgA antibody to *Salmonella typhi* LPS) were similar in the five sequentially collected specimens. Results for intake and output rates are shown in Fig. 2. These data confirm that peroral gut lavage provides a whole gut perfusion system for clinical research in gut immunology and inflammation.

5.3. Use of gut lavage for investigation of intestinal antibodies to bacterial endotoxin

5.3.1. Structure of endotoxin

In Edinburgh there has been considerable interest in the structure and immunogenicity of bacterial endotoxin, the properties of serum anti-endotoxin antibody, and the separation of antibodies, for therapeutic use, from blood donor plasma [37].

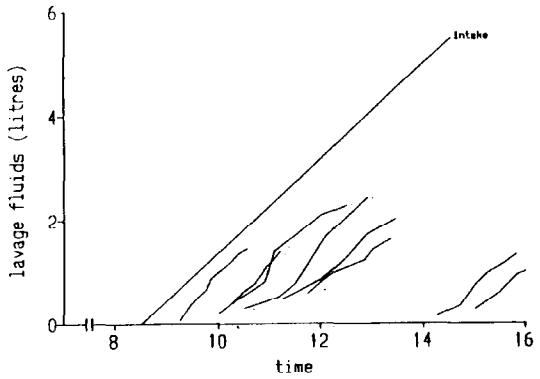

Fig. 2. Rates of intake and output of lavage fluid in eight patients; the lines are parallel, and as mentioned in the text, concentrations of several substances remained steady in all five specimens examined.

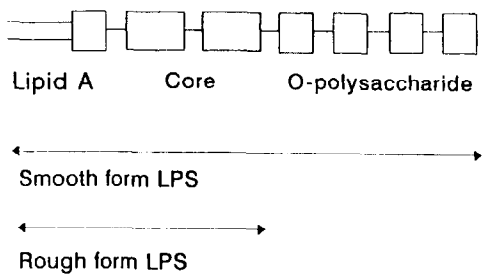

Fig. 3. Diagrammatic representation of lipopolysaccharide. The O-polysaccharide is a chain of heterogeneous length consisting of repeating oligosaccharide units which specify a vast range of serotype antigens. The outer region of the core oligosaccharide, which consists of five hexoses, has a degree of antigenic variability but is more conserved in the inner KDO/heptose region. Lipid A, the endotoxic part of the molecule, has a structure which is largely conserved in all gram-negative bacteria. If the O-polysaccharide is lost through mutation the LPS is termed "rough", compared to the "smooth" wild-type parent. Rough-form LPS is found naturally in some species of bacteria.

Fig. 3 illustrates the structure of LPS. Variations in the structure (and thus antigenicity) of the long carbohydrate O-polysaccharide chain allow serotyping of bacteria and aid their classification in bacterial taxonomy. However there is also antigenic cross-reactivity between gram-negative organisms, due to the existence of highly conserved core region antigens. "Rough" mutants of gram-negative bacteria lack the long polysaccharide chain and the core glycolipid antigens are exposed. ELISAs for IgG antibodies in blood donor sera have shown that Rc and Re antigens are immunodominant for man [38], and antibodies to these core LPS forms, reacting with many bacterial species, can neutralise endotoxin activity [39].

5.3.2. Serum and intestinal antibodies to LPS core antigens

We have measured IgA antibodies to LPS core antigens in matched specimens of serum and WGLF from 14 patients [32]. We found that, just as has been reported for IgG antibodies in serum, the Rc (and in some cases the Re) regions

were immunodominant for IgA antibody responses at the mucosal level. Results for antibodies to Rc in the 14 patients, expressed as empirical units, are shown in Fig. 4. Both in serum and in WGLF, levels of antibody were strikingly higher in Crohn's disease patients than in those with ulcerative colitis or normals. A patient with pouchitis had undetectable levels of serum antibody to this antigen but high intestinal antibody levels. Although ELISA data, being expressed as arbitrary units, are difficult to translate into absolute amounts of antibody, the values obtained show that intestinal secretion of IgA antibody to Rc LPS per day is equivalent to the IgA antibody content of around 500 ml blood.

5.3.3. Potential for active or passive immunisation

Clearly a vital question which could readily be answered by direct studies of mucosal immunity is whether patients with sepsis syndrome and multi-organ failure have adequate intestinal mucosal antibody to bacterial endotoxin, or whether there is transient or prolonged mucosal antibody failure in this situation.

Active gut immunisation prior to elective major surgery, general up-regulation of pre-existing low-titre anti-bacterial antibody, and even passive administration of antibody, via the gut, could well be effective, cheaper and safer than parenteral administration of anti-bacterial or anti-endotoxin antibody. It might not even be necessary to use human antibodies for such passive immunisation.

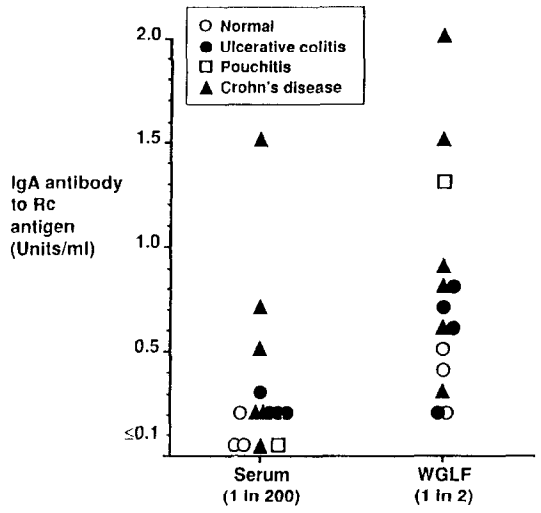

Fig. 4. Serum and WGLF IgA class antibodies to the Rc (rough mutant) antigen of *Salmonella minnesota*. Values given are arbitrary units of IgA antibody activity to the various antigens. Serum was diluted 1/200 and WGLF 1/2 before analysis.

5.4. Studies of gut immunity in healthy African children

5.4.1. Rationale for studying gut immunity in children

Many different bacterial, viral, protozoal and helminth pathogens are implicated in children with acute diarrhoeal diseases, and, in theory, the immune system of the gut could also contribute to these diseases in several ways. There may be intestinal immunodeficiency with failure to generate a protective immune response; local T cell and IgE-mediated immunity may produce the "innocent bystander" phenomenon–enteritis, colitis or malabsorption, as unavoidable accompaniments of many host-protective immune responses; and intestinal hypersensitivity to food antigens may be induced during an infection, with subsequent expression as food-sensitive enteropathy or colitis.

In view of the potential roles of intestinal immunodeficiency and hypersensitivity in the infection/diarrhoea/malnutrition cycle, a safe and ethical method to study intestinal immunity in children is needed. Guidelines for non-therapeutic research in children suggest that any investigative technique "subjects the child to no more than minimal risk as a result of his or her participation" [40]. Gut lavage seemed an ideal approach.

5.4.2. Assessment of gut immune status in African children

In collaboration with Dr Mary Hodges, Director of the St Andrew's Clinic for Children, and with the approval of the Ministry of Health

Research and Ethics Committee, Sierra Leone, gut lavage was successfully performed in 24 of 25 "normal" children from Freetown, Sierra Leone, with parental informed consent [41]. Study subjects were healthy children aged 6–9 years, recruited by a field assistant from the population of Brookfields. This area has a mixture of permanent housing of cement, and shanty town dwellings. The prevalence of intestinal parasites in children in this district was recently established as *Ascaris lumbricoides* 43%, Hookworm (probably *Necator americanus*) 21%, *Trichuris trichiura* 81%, *Strongyloides stercoralis* 5%, *Giardia lamblia* 27%, and *Cryptosporidium* 2% [42].

WGLF was treated with protease inhibitors, stored locally at −20°C, and transferred by air to Edinburgh for laboratory studies. These showed that no child had occult blood loss but four had evidence of protein-losing enteropathy. When compared with values for Scottish adults, WGLF from the Sierra Leonean children had significantly higher concentrations of IgA (Fig. 5) and IgM, and of IgA and IgM antibodies to dietary antigens and to *S. typhi* LPS. In three children, very low levels of IgA and IgA antibody were present. Clearly, substantial information on childrens intestinal immunity can be obtained by this method.

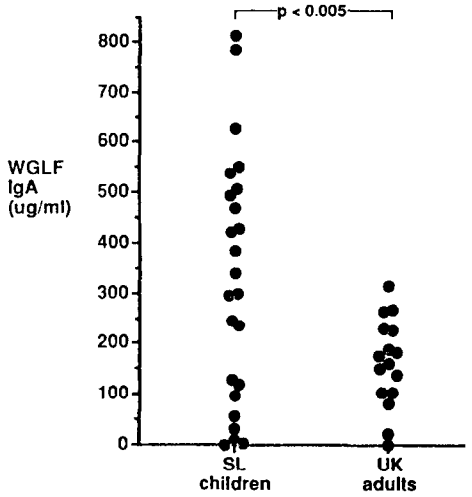

Fig. 5. Concentration of IgA in WGLF from 24 healthy Sierra Leonean children and 16 UK adults.

6. Studies of faeces are unhelpful

6.1. Possible analytical and sampling problems associated with the use of faeces for immunological testing

Increasing numbers of papers draw inferences on the immune functions of the gut from assays in faeces (e.g. Refs. [43–50]) but results obtained in this way have not generally been validated by comparison with determinations on directly collected gut secretions. When sensitive immunoassay and biochemical techniques are used on faecal extracts to assay immunoglobulins, other proteins, specific antibodies and cytokines, positive results are often obtained. Striking differences have been reported in the faecal concentrations of molecules of interest, when diseased cases (normally, diarrhoeal diseases) and controls are compared. These data would be sound if there is a standard rate of recovery of such molecules in faeces, irrespective of the presence or absence of intestinal inflammation, infection or allergic reaction.

Technical and analytical problems associated with the use of faeces are significant. There may be mechanical or biological interference with assays by substances present in faeces to a variable extent. The reference measure (often per gramme faeces) will be profoundly affected by faecal water content. Intestinal transit is also relevant – the time available within the lumen of the GI tract for molecules of interest to be destroyed by digestive enzymes and bacterial proteases. Published data on IgA illustrate the likely magnitude of these effects. Whereas the estimated intestinal production of IgA in humans (based on jejunal perfusion studies) is 40 mg/kg body weight (2.8 g) per day [18] reports of faecal IgA content are much lower, e.g. mean daily output of 58.5 mg, range 6–173 mg/day in 16 healthy adults [44].

An additional complicating factor is that some of the substances in faeces (and also in intestinal fluids) may be derived from plasma which has leaked into the gut lumen through ulcerated or inflamed sections of intestinal mucosa. We assess this in our work by assays of three plasma-derived molecules of different molecular weights,

IgG (some of which may be locally secreted), albumin and alpha-1-antitrypsin (A1AT) [26].

6.2. Comparison between faeces and whole gut lavage fluid

We have described in the previous section some of the evidence that gut lavage with a polyethylene glycol based solution is a whole gut perfusion system. By using WGLF as a standard, it is thus possible to measure the extent to which detection rates and recoveries of intestinal immunoglobulins, plasma-derived proteins and antibodies in faeces, truly reflect intestinal immune status. It should be borne in mind that the perfusion rate of gut lavage fluid is approximately 20 ml/min, equivalent to 29 l per day, whereas daily faecal output is normally in the range 50–200 g daily.

6.2.1. Patients and methods

We measured immunoglobulins, albumin, alpha-1-antitrypsin and isotype-specific antibodies in matched samples of faeces and whole gut lavage fluid [51]. Samples were obtained from 10 patients with active inflammatory bowel disease (activity confirmed by the objective measure of WGLF IgG concentration >10 μg/ml [27]) and 10 with other benign GI diseases. Gut lavage was performed as described above. Faecal samples were frozen at −70°C. Later, a portion of approximately 1 g was thawed, accurately weighed, homogenised in saline (10 ml per g), centrifuged at 20000 rpm for 15 min at 4°C, and 0.2 ml aliquots stored at −70°C for subsequent analysis. "Processed" faecal samples were prepared by treating saline extracts of faeces with the same series of protease inhibitors as WGLF.

6.2.2. Variable proportion of locally produced immunoglobulins and other proteins are detectable in faeces

Results were compared as estimated output per day, and by using haemoglobin as a common reference substance. For immunoglobulins, albumin and antibodies, the amount detected in faeces varied from <0.01 to 35.5% (based on estimated daily output) and <0.01 to 18.5% (based on haemoglobin) of the amount known to be produced in the gut from results of assays on WGLF; there were significantly higher rates of recovery in faecal specimens from patients with active gut inflammation than from other patients (Fig. 6).

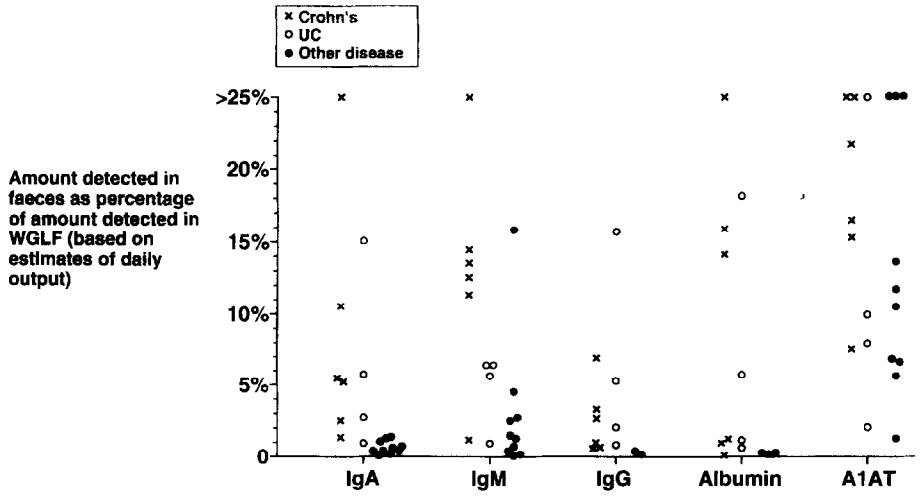

Fig. 6. Relative amounts of IgA, IgM, IgG, albumin and A1AT recovered from faeces of patients with IBD and with other benign diseases; estimates of daily outputs in faeces and in WGLF were calculated, and results expressed as percentage of the amount found in WGLF which can be detected in faeces. Data on specimens from non-IBD patients with barely detectable concentrations of IgG or albumin in WGLF, have been omitted.

We had observed that the detection of dietary protein antibodies in faeces was facilitated by processing the samples with protease inhibitors. However, in appropriately processed faeces, detection rates and titres of specific antibody were even lower than those for immunoreactive IgA (note that the assay used by us and most other workers for the total IgA ELISA detects immunoreactive alpha chain concentration, and thus measures partially degraded as well as intact IgA molecules). Quantitative ELISAs showed that IgA antibodies to OVA were present in WGLF from 19 of the 20 patients, with similar concentrations in IBD and non-IBD groups, whether expressed per ml WGLF, per mg IgA, per day or per mg Hb. As shown in Fig. 7, this was matched by the presence of antibody in processed faecal extracts in eight of the ten IBD cases, although in barely detectable titres (5–41 units/mg Hb) in four of these; antibody at very low titre (53 and 7 units/mg Hb) was detectable in processed faecal extracts of only two non-IBD patients, with diverticular disease and NSAID enteropathy. Taken alone, the results of these tests on faeces would have suggested abnormal mucosal immunity to ovalbumin in active IBD, whereas in fact there were similar levels of antibody in WGLF from the IBD and non-IBD cases.

Virtually identical results were obtained in studies of IgA antibodies to *S. typhi* LPS in ten patients. Assays on WGLF were positive in nine of the ten specimens studied, ranging from 1.2–4.2 units/ml WGLF in four IBD patients, and 2.1–5.6 units/ml WGLF in five non-IBD cases (with one non-IBD case negative). In contrast, although antibody was detected in the faecal extracts of three of the four IBD patients, 1.0–1.2 units/g, there was a positive result in only one of the six non-IBD patients, at the low level of 0.6 units/g.

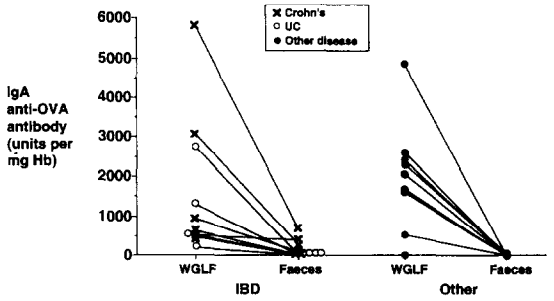

Fig. 7. Concentrations of IgA anti-ovalbumin antibody in WGLF and faeces (expressed per mg Hb) from patients with IBD and with other benign diseases.

7. Examples of the use of gut lavage to study mucosal immunity to oral vaccines

7.1. Determinants of mucosal immune response to vaccination

It is now almost axiomatic that vaccination against enteric infections such as typhoid fever should be via the portal of entry of the infectious agents. Gut antibodies may provide antibacterial protection by two mechanisms: direct action on bacteria, which may result in immobilization, agglutination, or prevention of adherence to the mucosa; and combination with bacterial products such as toxins or enzymes which cause inactivation and help in their destruction by proteolytic enzymes.

Theoretically, many factors may influence the mucosal antibody response to a vaccine.

The route of administration is undoubtedly important. Oral administration produces a more vigorous mucosal immune response than the parenteral route [52–54]; indeed parenteral immunization may actually suppress mucosal immunity [55,56]. Previous exposure to the same or a cross-reacting antigen may prime the mucosal immune system [57,58], and live vaccines generally induce produce stronger mucosal immune response than do killed vaccines [52,59].

There are remarkably few data on this subject in man, whether for healthy volunteers or those with potentially aberrant immunity attributable to age, nutrient deficiency, co-existing disease or genetic make-up. As part of a programme of research on the regulation of immunity and hypersensitivity in the human GI tract, we have conducted two studies, using whole gut lavage, on mucosal antibody production after oral immunisation.

7.2. Immune responses to oral cholera vaccine in coeliac disease patients and controls

Cholera toxin (CT) is composed of one A subunit and five B subunits held together in a pentamer. The toxin B subunit binds to a membrane receptor (ganglioside GM1) of gut epithelial cells, and the enterotoxic effect is exerted through the A subunit.

Protective immunity against cholera is dependent on stimulation of the intestinal immune system, in particular secretory IgA antibodies [58] IgA anti-bacterial as well as antitoxic intestinal antibodies are associated with protection against cholera infection, and both are induced by natural infection or oral vaccination. Antitoxic immunity as conferred by locally produced IgA antibodies probably exert their protective function by neutralising the toxin before it binds to the epithelial cell.

A new oral cholera vaccine (combined killed whole-cell/ B subunit), has been developed for use in endemic areas. This oral combined vaccine evokes high levels of specific intestinal antibodies along with antitoxic protection in endemic populations as well as in human volunteers [58,60].

Given the profound disturbances in systemic and mucosal immunity in patients with coeliac disease, it might be expected that their immune response to vaccines may differ from those of healthy volunteers. To date, no study has addressed this issue.

7.2.1. Subjects and methods

Seven healthy volunteers and nine untreated coeliac patients were studied, age range 24–46 years. All coeliac patients had had a recent jejunal biopsy showing subtotal or severe partial villus atrophy, and all were taking a normal (i.e., gluten-containing) diet.

The oral cholera vaccine used was a combined B subunit-whole cell killed vaccine, a gift of Professor Jan Holmgren (Gothenburg, Sweden). Each dose of vaccine contained 1 mg of B subunit and 10^{11} killed vibrios. The vaccine was given mixed with 150 ml of a sodium bicarbonate solution.

Following baseline collections of serum and gut lavage fluid, three controls and four coeliacs were given cholera vaccine on days 1 and 15; collection of serum and gut lavage fluid was repeated on day 25. A further three controls and five coeliacs were given vaccine on days 1, 15 and 29, with collection of serum and gut lavage fluid on day 39. In fact, results were similar for the 2 and 3 dose schedules and so results were combined.

Isotype-specific antibodies to cholera toxin were assayed in WGLF (IgA and IgM) and serum (IgA and IgG). The method used was a minor adaptation of a published "G_{M1}" method [20); precoating the ELISA plates with this protein ensures optimal binding of the cholera toxin B-subunit. Results were expressed semi-quantitatively as% of a reference standard which differed for each isotype.

7.2.2. Results and interpretation – intestinal antibodies (Fig. 8)

Before immunisation, both IgM and IgA antibody levels in WGLF were significantly higher in the coeliac patients (both $p < 0.03$) compared to controls.

Immunisation resulted in an increase in WGLF IgA antibody activity in five of seven controls, and in five of nine coeliacs. However, since the pre-vaccination levels of antibody differed, no conclusion can be drawn as to any possible intrinsic difference in gut immune capacity between coeliacs and others. For WGLF IgM antibodies, immunisation resulted in an increase in three of seven controls and five of nine coeliacs. Post-immunisation antibody levels in controls were, however, generally low (all <0.2). Post-immunisation IgM antibody levels were significantly higher in the coeliac patients ($p < 0.003$).

Why should coeliac patients who have never had clinical cholera or been exposed to cholera vaccine have high levels of specific intestinal antibodies? The most likely explanation is that in coeliac disease, there is a generalised up-regulation of intestinal humoral immune response, with increased levels of immunoglobulin (particularly IgM) and antibodies to a variety of dietary antigens [61]; it is likely that humoral immunity to other luminal antigens (such as microbial agents) is also increased in coeliac disease. This

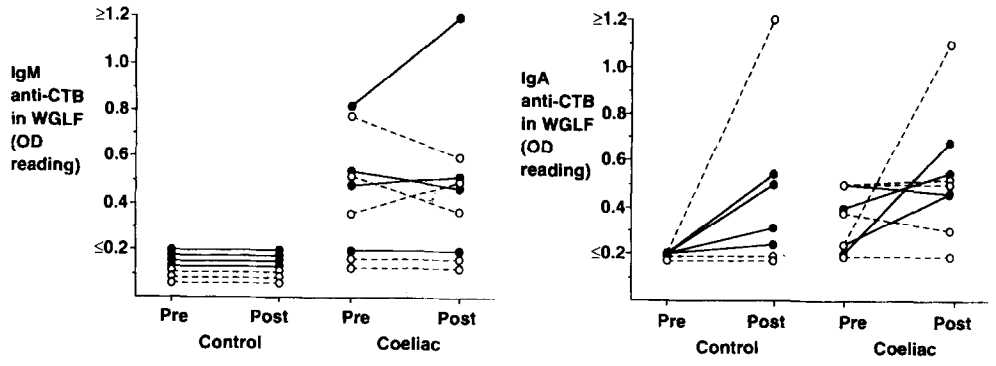

Fig. 8. Gut lavage fluid IgA and IgM antibody responses to cholera toxin in coeliacs and controls.

being the case, coeliac patients would have high titres of intestinal antibodies to many microbial antigens including *E. coli* toxin which may have antigenic cross-reactivity with the cholera toxin B subunit.

7.2.3. Results and interpretation – serum antibodies

In serum, immunisation resulted in an increase in IgG antibody in six of seven controls, and seven of nine coeliacs, and of IgA antibody in six of seven controls, and seven of nine coeliacs. In general, serum responses tended to mirror intestinal responses. However, an IgA serum response was seen in one control patient and two coeliac patients who did not have an intestinal IgA response. There were no significant differences between controls and coeliacs in levels of either pre or post-immunisation serum IgG or IgA anti-toxin antibodies.

Serum anti-toxin responses seemed in general to reflect the intestinal immune responses; this is in accord with the study reported by Jertborn et al. [60] who found that serum antibody responses to oral cholera vaccine had a predictive accuracy of about 80% for reflecting the intestinal immune response. They pointed out that while measurement of serum antibody was a useful predictor of intestinal immune response in large field trials, it was of little use in the *individual* subject. It is also noteworthy, as discussed above, that CT is an unusually potent immunogen, and that feeding of many other antigens does not elicit serum antibodies, in contrast to CT.

7.3. Effects of smoking on intestinal antibodies elicited by the oral typhoid vacine Ty21a in healthy UK adults

The live attenuated oral typhoid vaccine Ty21a lacks the capsular antigen Vi which is the virulence factor in pathogenic strains of *S. typhi* [62]. When given as an oral typhoid vaccine (five to eight doses containing $3–10 \times 10^{10}$ viable organisms) to healthy volunteers from non-endemic area, Ty21a provided 87% clinical protection against experimental challenge with 10^5 virulent *S. typhi* [63].

The vaccine has proved to be safe, with mild, if any, adverse reactions, and genetically stable. Large-scale field trials carried out in Egypt, Chile and Indonesia measuring its protective efficacy have shown significant although variable levels of protection against typhoid fever [64–67]. The variation in the rates of protection was attributed to the differences in the formulations, vaccination schedules and the incidence rates of infection in these countries.

The best published data on the capacity of the vaccine strain to induce local mucosal immunity are those of Forrest [15,22,68–70], who studied healthy Australian volunteers and specifically excluded individuals with a history of salmonella infection or food poisoning. However, most of his volunteers received doses of the organism one or two logs greater than those used in recent trials. He studied a variety of formulations, dosages and timing of administration using jejunal aspirate as a material for the study, and

established that 3 weeks was the optimal timing for studying the intestinal immune responses to this vaccine. We have taken this work further by investigating the effect of smoking on gut immunity by using the preparation of oral typhoid vaccine Ty21a, recently licenced for use in the UK, as an immunogen.

7.3.1. Subjects and methods

We recruited 22 healthy adult volunteers, age range 22–44 years. Subjects who drank more than 20 units of alcohol per week were excluded. Only subjects who were either heavy smokers (more than 20 cigarette per day for the last 3 years) or lifelong non-smokers, were selected. Subjects had gut lavage and baseline serum sample collected, and were then given three enteric-coated capsules of the oral typhoid vaccine Ty21a, with each capsule containing 2×10^9 live *S. typhi* organims, to be taken on days 1, 3 and 5, not with food. 3 weeks WGLF and serum were again collected. IgA anti-*S. typhi* lipopolysaccharide (LPS) antibodies were measured in WGLF and serum by a quantitative ELISA. Serum with a high titre (expressed as arbitrary units) was used as a reference standard and the results of the test samples were expressed as units/ml and also, for WGLF, as units/mg IgA.

7.3.2. Results – intestinal antibody responses (Fig. 9)

The vaccine Ty21a was well-tolerated and there were no significant differences between pre- and post-vaccination concentrations of total IgA or IgM in WGLF ($p = 0.63$). Antibody results were considered to be significantly increased or decreased by vaccination if the changes were found to be more than 20% greater or less than the pre-immunisation values (the inter-assay coefficient of variation of the ELISA used was 15–20%). There was a significant increase in specific IgA antibody in WGLF specimens of 14 out of 22 volunteers (63.6%) at 3 weeks after vaccination with Ty21a. However, there was a significant decrease in specific IgA antibody in four volunteers (18.2%). A further four volunteers had no significant change in specific IgA antibody levels. Some of the volunteers had a rise in serum anti-*S. typhi* LPS IgG and IgA antibodies, but there was no significant relationship between the changes in serum and those in WGLF.

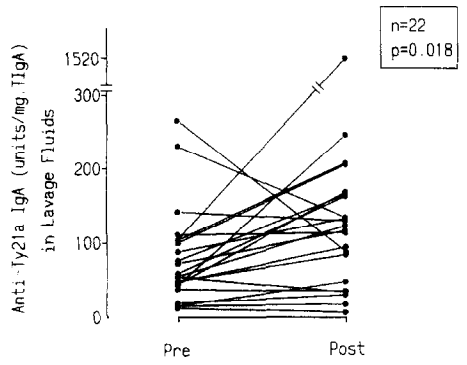

Fig. 9. IgA class antibodies to *Salmonella typhi* LPS in whole gut lavage fluid from four healthy volunteers, before and at 3 weeks after vaccination with three doses of oral Ty21a vaccine (given on days 1, 3 and 5)

Finally, there were no differences in any of the parameters measured when non-smokers and heavy smokers were compared.

8. Conclusions

Methods for safe and non-invasive investigation of gut mucosal immunity, including immunity to oral vaccines, are now available. We suggest that these techniques can be used to provide information critical to the understanding of the pathogenesis of diarrhoeal disease morbidity, and necessary for the design and delivery of effective oral immunisation. International and rural/urban comparisons will be of great interest, as will studies of the heterogeneity of intestinal immunity *within* members of a local community in the developing or developed world. Such investigations might address the pathogenesis and clinical significance of mucosal IgA deficiency; the characteristics of children or adults who, within the same highly contaminated environment, remain pathogen-free in contrast to the majority who are frequently re-infected or are chronic carriers; nutrition, immunity, host-parasite interactions and malabsorption in giardiasis; the relative importance of losses of pro-

tein and haemoglobin from the gut, as compared to other mechanisms, in children with anaemia and hypoproteinaemia; and factors that may influence intestinal antibody responses to peroral vaccines for cholera, typhoid and rotavirus.

References

[1] Ogra, P.L., Mestecky, J., Lamm, M.E., Strober, W., McGhee, J.R. and Bienenstock, J. (1994) Handbook of Mucosal Immunology, Academic Press, New York.

[2] Lamont, A.G., Gordon, M. and Ferguson, A. (1988) T lymphocyte function in protein deprived mice. Clin. Exp. Immunol. 72, 113–117.

[3] Troncone, R. and Ferguson, A. (1988) Gliadin presented via the gut induces oral tolerance in mice. Clin. Exp. Immunol. 72, 284–287.

[4] Strobel, S. and Ferguson, A. (1987) Persistence of oral tolerance in mice fed ovalbumin is different for humoral and cell-mediated immune responses. Immunology 60, 317–318.

[5] Strobel, S. and Ferguson, A. (1984) Immune responses to fed protein antigens in mice. 3. Systemic tolerance or priming is related to age at which antigen is first encountered. Pediatr. Res. 18, 588–593.

[6] Kay, R.A. and Ferguson, A. (1989) Systemic delayed-type hypersensitivity to cholera toxin and a detoxified derivative. Clin. Exp. Immunol. 76, 111–116.

[7] Kay, R.A. and Ferguson, A. (1989) The immunological consequences of feeding cholera toxin. I. Feeding cholera toxin suppresses the induction of systemic delayed-type hypersensitivity but not humoral immunity. Immunology 66, 410–415.

[8] Kay, R.A. and Ferguson, A. (1989) The immunological consequences of feeding cholera toxin. II. Mechanisms responsible for the induction of oral tolerance for DTH. Immunology 66, 416–421.

[9] Mowat, A.McI. and Ferguson, A. (1982) Intraepithelial lymphocyte count and crypt hyperplasia measure the mucosal component of the graft-versus-host reaction in mouse small intestine. Gastroenterology 83, 417–423.

[10] Husby, J., Elson, C.O., Moldoveanu, Z. and Mestecky, J. (1994) Oral tolerance in humans. T Cell but not B cell tolerance to a soluble protein antigen. J. Immunol. 152, 4663–4670.

[11] Husband, A.J., Monie, H.J. and Gowans, J.L. (1977) The natural history of the cells producing IgA in the gut. Ciba. Found. Symp. 46, 29–54.

[12] Ghosh, S., Ferguson A (1994) Mucosal immunology research design should address not just cytokines and cells, but also clinical data and controls. Editorial Review. Clin. Exp. Immunol. 96, 377–378.

[13] Czerkinsky, C.C., Nilsson L.-A., Nygren, H., Ouchterlony, O. and Tarkowski, A. (1983) A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J. Immunol. Methods 65, 109–121.

[14] Forrest, B.D. (1988) Identification of an intestinal immune response using peripheral blood lymphocytes. Lancet i, 81–83.

[15] Forrest, B.D., LaBrooy, J.T., Beyer, L., Dearlove, C.E. and Shearman, D.J.C (1991) The human humoral immune response to *Salmonella typhi* Ty21a. J. Infect. Dis. 163, 336–345.

[16] Kelly, C.P., Pothoulakis, C., Orellana, J. and Lamont, J.T. (1992) Human colonic aspirates containing immunoglobulin A antibody to *clostridium difficile* toxin A inhibit toxin A-receptor binding. Gastroenterology 102, 35–40.

[17] O'Mahony, S., Arranz, E., Barton, J.R. and Ferguson, A. (1991) Dissociation between systemic and mucosal humoral immune responses in coeliac disease. Gut 32, 29–35.

[18] Conley, M.E. and Delacroix, D.L. (1987) Intravascular and mucosal immunoglobulin A. Two separate but related systems of immune defence? Ann. Intern. Med. 106, 892–899.

[19] Cellier, C., Sahmoud, T., Froguel, E., Adenis, A., Belaiche, J., Bretagne J.-F., Florent, C., Bouvry, M., Mary J.-Y. and Modigliani, R. (1994) Correlations between clinical activity, endoscopic severity, and biological parameters in colonic or ileocolonic Crohn's disease. A prospective multicentre study of 121 cases. Gut 35, 231–5.

[20] Gaspari, M.M., Brennan, P.T., Soloman, S.M. and Elson, C.O. (1988) A method of obtaining, processing, and analysing human intestinal secretions for antibody content. J. Immunol. Methods 110, 85–91.

[21] O'Mahony, S., Barton, J.R., Crichton, S. and Ferguson, A. (1990) Appraisal of gut lavage in the study of intestinal humoral immunity. Gut 31, 1341–1344.

[22] Forrest, B.D. (1992) Effects of sample processing on the measurement of specific intestinal IgA immune responses. Vaccine 10, 802–805.

[23] Svennerholm A.-M., Sack, D.A., Holmgren, J. and Bardhan, P.K. (1982) Intestinal antibody responses after immunization with cholera B subunit. Lancet i, 305–308.

[24] Arranz, E., O'Mahony, S., Barton, J.R. and Ferguson, A. (1992) Immunosenescence and mucosal immunity: significant effects of old age on serum and secretory IgA concentrations and on intraepithelial lymphocyte counts. Gut 33, 882–86.

[25] O'Mahony, S., Choudari, C.P., Barton, J.R., Walker, S., Ferguson A (1991) Gut lavage fluid proteins as markers of activity of inflammatory bowel disease. Scand. J. Gastroenterol. 26, 940–944.

[26] Brydon, W.G., Choudari, C.P. and Ferguson, A. (1993) Relative specificity for active inflammatory bowel disease of plasma-derived proteins in gut lavage fluid. Eur. J. Gastroenterol. Hepatol. 5, 269–273.

[27] Choudari, C.P., O'Mahony, S., Brydon, G., Mwantembe, O. and Ferguson, A. (1993) Concentrations of immunoglobulin G, albumin and alpha-a-antitrypsin in whole gut lavage fluid: objective measures of disease activity in

inflammatory bowel disease. Gastroenterology 104, 1064–1071.

[28] Brydon, W.G. and Ferguson, A. (1992) Haemoglobin in gut lavage fluid as a measure of gastro-intestinal blood loss. Lancet 340, 1381–1382.

[29] Ferguson, A., Ghosh, S., Handy, L.M., Choudari, C., Mwantembe, O. and McIntyre, M.A. (1994) Analysis of disease distribution, activity and complications in the patient with inflammatory bowel disease. Scand. J. Gastroenterol. 29 (Suppl. 203), 15–19.

[30] Handy, L.M., Ghosh, S. and Ferguson A. (1995) Investigation of neutrophil migration into the gut by cytology of whole gut lavage fluid. Eur. J. Gastroenterol. Hepatol. 7, 53–58.

[31] O'Mahony, S., Anderson, N., Nuki, G. and Ferguson, A. (1992) Systemic and mucosal antibodies to klebsiella in ankylosing spondylitis and Crohn's disease. Ann. Rheum. Dis. 51, 1296–1300.

[32] Ferguson, A., Sallam, J., McLintock, L., Croft, N., Poxton I (1994) The gut as an immune organ: Intestinal anti-endotoxin antibodies. In: J.M. Kinney and H.N. Tucker (Eds), Organ Metabolism and Nutrition: Ideas for Future Critical Care, Raven Press, New York, pp. 231–244.

[33] Sallam, J. and Ferguson, A. (1992) Mucosal immunity to oral vaccines. Lancet 339, 179.

[34] Ferguson, A. and Mwantembe, O. (1995) Intestinal lavage test of gut inflammation and immunity. In: S. Auricchio, R. Troncone and A. Ferguson (Eds), Mucosal Immunity in the Gut Epithelium, Karger, Basel, in press.

[35] Sallam, J. (1994) Intestinal humoral immunity in man: IgA and anti-salmonella antibodies. Thesis, University of Edinburgh.

[36] Ferguson, A., Ghosh, S. and Brydon, W.G. (1995) Use of whole gut lavage fluid to measure occult GI bleeding and protein-losing enteropathy. Z. gastroenterol. in press.

[37] Barclay, G.R. (1990) Antibodies to endotoxin in health and disease. Rev. Med. Microbiol. 1, 133–142.

[38] Barclay, G.R. and Scott, B.B. (1987) Serological relationships between *Escherichia coli* and *Salmonella* smooth- and rough-mutant lipoplysaccharides as revealed by enzyme-linked immunosorbent assay for human immunoglobulin G antiendotoxin antibodies. Infect. Immun. 55, 2706–2714.

[39] Di Padova, F., Brade, H., Barclay, G.R., Poxton, I.R., Liehl, E., Schuetze, E., Kocher, H.P., Ramsay, G., Schreier, M.H., McClelland, D.B.L. and Rietschel, E.T. (1993) A broadly cross-protective monoclonal antibody binding to *Escherichia coli* and *Salmonella* lipopolysaccharide. Infect. Immun. 61, 3863–3872.

[40] Working Party on Research on Children, The ethical conduct of research on children (1991) Medical Research Council.

[41] Hodges, M., Kingstone, K., Brydon, W.G., Sallam, J. and Ferguson, A. (1994) Use of gut lavage fluid to measure intestinal immunity in healthy Sierra Leonean children. J. Pediatr. Gastroenterol. Nutr. 19, 65–70.

[42] Webster, J., Hodges, M.H., Crompton, D.W.T and Walters, D.E. (1990) Intestinal parasite infections in children from Freetown, Sierra Leone. J. Sierra Leone Med. Dent. Assoc. 5, 144–155.

[43] Haneberg, B. and Tonder, O. (1973) Immunoglobulins and other serum proteins in feces from infants and children. Scand. J. Immunol. 2, 375–383.

[44] Meillet, D., Raichvarg, D., Tallet, F., Savel, J., Yonger, J. and Gobert, J.G. (1987) Measurement of total, nomomeric and polymeric IgA in human faeces by electro-immunodiffusion. Clin. Exp. Immunol. 69, 142–147.

[45] Koutras, A.K. and Vigorita, V.J. (1989) Fecal secretory immunoglobulin A in breast milk versus formula feeding in early infancy. J. Pediatr. Gastroenterol. Nutr. 9, 58–61.

[46] Saha, K., Sharma, S., Chopra, K. and Dua, N. (1990) Raised stool and serum IgA levels in undernourished infants with chronic diarrhoea and associated parasitic infestations. J. Trop. Pediatr. 36, 69–74.

[47] Sasai, K., Furukawa, S., Sugawara, T., Kaneko, K., Baba, M. and Yabuta, K. (1992) IgE levels in faecal extracts of patients with food allergy. Allergy 47, 594–598.

[48] Cancellieri, V. and Fara, G.M. (1985) Demonstration of specific IgA in human feces after immunization with live Ty21a *Salmonella typhi* vaccine. J. Infect. Dis. 151, 482–484.

[49] Stapleton, J.T., Lange, D.K., LeDuc, J.W., Binn, L.N., Jansen, R.W. and Lemon, S.M. (1991) The role of secretory immunity in hepatitis A virus infection. J. Infect. Dis. 163, 7–11.

[50] Harendra de Silva, D.G., Mendis, L.N., Alexander, G.J.M, Candy, D.C.A, Chart, H. and Rowe, B. (1993) Concentrations of interleukin 6 and tumour necrosis factor in serum and stools of children with *Shigella dysenteriae* 1 infection. Gut 34, 194–198.

[51] Ferguson, A., Croft, N. and Humphreys, K. (1995) Technical Report: Results of immunological tests on faecal extracts are likely to be extremely misleading. Clin. Exp. Immunol. 99, 70–75.

[52] Ogra, P., Karzon, D. and Righthand, F. (1968) Immunoglobulin response in serum and secretions after immunization with live and inactivated polio vaccine and natural infection. N. Engl. J. Med. 279, 893–900.

[53] Lange, S. and Holmgren, J. (1978) Protective antitoxic Cholera immunity in mice: influence of route and number of immunizations and mode of action of protective antibodies. Acta Pathol. Microbiol. Scand. Sect. 86, 145–152.

[54] Svennerholm A.-M., Hanson, L. and Holmgren, J. (1980) Different secretory immunoglobulin A antibody responses to cholera vaccination in Swedish and Pakistani women. Infect. Immun. 30, 427–430.

[55] Pierce, N. (1984) Induction of optimal mucosal antibody responses: effects of age, immunization route(s) and dosing schedules in rats. Infect. Immun. 43, 341–346.

[56] Pierce, N. and Koster, F. (1980) Priming and suppression of the intestinal immune response to cholera

toxoid/toxin by parenteral toxoid in rats. J. Immunol. 124, 307–311.

[57] Svennerholm A.-M., Holmgren, J. and Hanson, L. (1977) Boosting of secretory IgA antibody responses in man by parenteral Cholera vaccination. Scand. J. Immunol. 6, 1345.

[58] Svennerholm A.-M., Gothefors, L. and Sack, D. (1984) Local and systemic antibody responses and immunological memory in humans after immunization with cholera B subunit by different routes. Bull. WHO 62, 909–918.

[59] Levine, M., Ferreccio, C. and Black, R. (1989) Progress in vaccines against typhoid fever. Rev Infect. Dis. ii (Suppl. 3), S552–S567.

[60] Jertborn, M., Svennerholm A.-M. and Holmgren J. (1984) Gut mucosal, salivary and serum antitoxic and antibacterial antibody response in Swedes after oral immunization with B subunit-whole cell Cholera vaccine. Int. Arch. Allergy Appl. Immunol. 75, 38–43.

[61] Arranz, E. and Ferguson, A. (1993) Intestinal antibody pattern of coeliac disease: occurrence in patients with normal jejunal biopsy histology. Gastroenterology 104, 1263–1272.

[62] Silva, B., Gonzalez, C. and Mora, G. (1978) Genetic characteristics of the *Salmonella typhi* strain Ty21a vaccine. J. Infect. Dis. 155, 1077–1078.

[63] Gilman, R., Hornick, R. and Woodward, W. (1977) Evaluation of a UDP-glucose-4-epimeraseless mutant of *Salmonella typhi* as a live oral vaccine. J. Infect. Dis. 136, 717–723.

[64] Wahdan, M., Serie, C. and Cerisier, Y. (1982) A controlled field trial of live *Salmonella typhi* strain Ty21a oral vaccine against typhoid: Three-year results. J. Infect. Dis. 145, 292–295.

[65] Wahdan, M., Serie, C. and Germanier, R. (1980) A controlled field trial of live oral typhoid vaccine Ty21a. Bull. WHO 58, 469–474.

[66] Levine, M., Ferreccio, C. and Black, R. (1987) Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formation. Lancet i, 1049–1052.

[67] Simanjuntak, C., Paleologo, F. and Punjabi, N. (1991) Oral immunization against typhoid fever in Indonesia with Ty21a vaccine. Lancet 338, 1055–1059.

[68] Forrest, B., LaBrooy, J. and Attridge, S. (1989) A candidate live oral typhoid/cholera hybrid vaccine is immunogenic in humans. J. Infect. 159, 145–146.

[69] Forrest, B. (1992) Impairment of immunogenicity of *Salmonella typhi* Ty21a due to pre-existing cross-reacting intestinal antibodies. J. Infect. Dis. 166, 210–212.

[70] Forrest, B., LaBrooy, J. and Dearlove, C. (1992) Effect of parenteral immuninzation on the intestinal immune response to *Salmonella typhi* Ty21a. Infect. Immun. 60, 465–471.