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Intestinal and serum antibody in coeliac disease: a comparison using ELISA

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SUMMARY

Intestinal and serum antibody to antigens derived from gluten and other food proteins in 16 children with coeliac disease and 15 control subjects was measured using an enzyme-linked immunosorbent assay (ELISA). High concentrations of antibody to gluten antigens were found in children with coeliac disease who were on a diet which contained gluten. This antibody was predominantly in the IgA and IgM classes in intestinal fluid, and in the IgG and IgA classes in serum. When coeliac children transferred to a gluten-free diet for 6 months or more, anti-gluten antibody fell much more rapidly in serum than in intestinal fluid. Although no single measure of antibody, in any immunoglobulin class, to a gluten-derived antigen proved sufficiently discriminating to be suggested as a diagnostic test for coeliac disease, serum antibody, particularly in the IgA class, may be of value in following the progress of patients and in assessing their adherence to a gluten-free diet.

Keywords coeliac anti-gluten antibody intestinal antibody gluten-free diet

INTRODUCTION

Numerous recent studies have focused on measurements of immunity in the peripheral blood of patients with coeliac disease (Burgin-Wolff *et al.*, 1983; O'Farrelly *et al.*, 1983; Weiss *et al.*, 1983; Unsworth *et al.*, 1981; Savilahti *et al.*, 1983; O'Farrelly *et al.*, 1982; Bullen & Losowsky, 1976). In contrast, information on specific intestinal immune responses in this condition is limited. The studies which have examined this facet of the immune response in coeliac disease have used antibody assays (such as precipitin tests in agarose) which are both relatively insensitive and unable to distinguish between the various immunoglobulin classes of antibody (Katz, Cantor & Herskovic, 1968; Herskovic *et al.*, 1968; Ferguson & Carswell, 1972), or have measured antibody synthesis by a limited number of intestinal biopsies cultured *in vitro* (Loeb *et al.*, 1972; Falchuk & Strober, 1974).

The need for a sensitive assay to examine the intestinal antibody response in coeliac disease prompted this study. An enzyme linked immunosorbent assay (ELISA) was used to measure antibody in the intestinal aspirate and serum of children with coeliac disease to three antigens derived from gluten and two other food protein antigens.

MATERIALS AND METHODS

Patients and controls. Sixteen subjects (eight males and eight females; median age 2 years 6 months, range 11 months to 14 years), in whom an unequivocal diagnosis of coeliac disease was

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made according to the criteria of the European Society of Paediatric Gastroenterology and Nutrition (McNeish *et al.*, 1979), were studied while on a diet containing gluten. In ten of them the study was repeated at a time when they had been on a gluten-free diet for at least 6 months. With these patients, the histology of the biopsies improved after the gluten-free diet and deteriorated when gluten was re-introduced (Table 1). The controls were 15 children (nine males and six females; median age: 2 years 6 months, range 4 months to 15 years) who were subjected to intestinal biopsy because of the possibility that they had coeliac disease but in whom the jejunal histology excluded this diagnosis. The diagnoses are shown in Table 2. Chronic non-specific diarrhoea (CNSD) was defined according to the criteria of Davidson & Wasserman (1966). Post-gastroenteritis syndrome (PGS) was defined as diarrhoea persisting for greater than 2 weeks after acute enteritis.

Sera and intestinal aspirates were obtained from these subjects at the time they presented for intestinal biopsy and were stored at −70°C until assayed. The intestinal aspirate was obtained through a tube attached to a Watson Paediatric Intestinal Biopsy Capsule, which was positioned with the aid of fluoroscopy at the duodeno-jejunal flexure (Ford, 1981). Only intestinal fluid with a pH greater than 6 was used in these studies.

Enzyme-linked immunosorbent assay. The three gluten-derived antigens used in the assay were gliadin (a complex mixture of alcohol-soluble wheat proteins; Sigma Chemicals), Frazer's fraction III (Frazer *et al.*, 1959; prepared by peptic tryptic digestion of gluten) and Cornell's fraction 9 (Cornell & Townley, 1973; prepared by ion-exchange chromatography from a peptic tryptic digest of gliadin). These three cereal antigens have the capacity of undigested gluten to damage the intestinal mucosa of coeliac patients. However, as the digested fractions are relatively simple substances, it was thought that immune responses to these peptides may be the most relevant to the pathogenesis of coeliac disease. The food antigens used in the assays were beta lactoglobulin (β-LG) (present in milk) and bovine serum albumin (BSA) (present in milk and meat) (Sigma Chemicals).

The assay used was an enzyme-linked immunoabsorbent assay described previously (Hohmann *et al.*, 1983), using microtitre plates (Costar) in which the wells had been coated with antigen at a concentration of 5 μg/ml in phosphate-buffered saline except in the case of gliadin. Gliadin was attached to the plates by dissolving it in 70% ethanol at the same concentration of 5 μg/ml and evaporating the solvent to dryness in the microtitre wells. 100 microlitre aliquots of serial dilutions of serum or intestinal fluid were added to each well and incubated for 2 h at 20°C. In the case of the intestinal fluid this initial incubation was carried out in the presence of 2 mM phenyl-methyl-

Table 1. The histological grading of intestinal lesions in biopsies taken from coeliac children at diagnosis, on a gluten-free diet and after challenge

		Histological grading	
Patient	At diagnosis	On a gluten-free diet	After challenge
A	4	1	4
B	3	1	3
C	4	1	3
D	4	2	3
E	4	1	3
F	NA	2	4
G	4	1	3—4
H	4	2	3
I	NA	1	3
J	3	1	2

Histological grading: 1, normal; 2, mild lesion (partial villous atrophy); 3, moderate (severe partial villous atrophy); 4, severe (subtotal villous atrophy).

NA, not available.

Table 2. The clinical diagnoses of the control group

Number of patients	Diagnoses
7	CNSD
3	Short stature
2	PGS
1	Cow's milk protein intolerance
2	Previous diagnosis of coeliac disease made on clinical grounds disproven

PGS defined as diarrhoea persisting for greater than 2 weeks after acute enteritis.

CNSD defined according to the criteria of Davidson & Wasserman (1966).

sulphonyl fluoride (PMSF) which is a protease inhibitor that markedly inhibits degradation of both antigen and antibody by intestinal enzymes (Hohmann *et al.*, 1983). IgA, IgM and IgG antibody binding to the wells was quantified using class specific monoclonal antibodies (Bethesda Research Laboratories), alkaline phosphatase linked to anti-mouse immunoglobulin (Dako immunoglobulins) and substrate (Para-nitrophenyl phosphate) in series, with washing in between. The ELISA titre was assigned as the reciprocal of the highest dilution of serum or aspirate which gave an O.D. 405 of 0·15 (Hohmann *et al.*, 1983).

Statistical analysis of the difference between levels of antibody measured in the various groups used a 'likelihood ratio' test.

RESULTS

Sera and intestinal aspirates from 16 children with coeliac disease and 15 control subjects were examined for the presence of antibody to the five dietary antigens. In the ELISA assays carried out, the pattern of immune reactivity to the three wheat antigens, gliadin, Frazer's fraction III and Cornell's fraction 9, was similar, as was reactivity to the two other food proteins, β-LG and BSA. Hence, only the data for Frazer's fraction III and β-LG have been presented here in detail, being representatives of one gluten-derived and one non-wheat antigen. As is to be expected from the relative amounts of the main immunoglobulin classes in intestinal fluid and in serum, antibody in intestinal secretions was mainly in the IgA and IgM classes whereas IgG antibody predominated in the serum.

Intestinal antibodies to gluten derived antigens. Intestinal antibody to either the gluten-derived antigens (Frazer's fraction III) or other food proteins (β-LG) was not detectable in most of the control subjects (Fig. 1). In contrast, high levels of antibody to Frazer's fraction III were found in the aspirates of patients with coeliac disease. Intestinal antibody to β-LG was also present in about half of the coeliac children on a normal, gluten-containing diet (Fig. 1). While coeliac children were on a gluten-free diet, intestinal anti-gluten antibody levels appeared to decline slightly, but this did not reach statistical significance. No consistent pattern of change was noted in gut antibody to the non-wheat antigens in these children when gluten was excluded from their diet (Figs 1 and 3).

In addition to the measurements of specific antibody, the concentrations of total immunoglobulin were estimated in a proportion of the subjects using the Mancini single radial immunodiffusion technique (LaBrooy *et al.*, 1980). The mean ($\pm$s.d.) immunoglobulin concentrations found in the aspirates of six control subjects were IgG, 4·9$\pm$3·8 mg%, IgA 13·5$\pm$7·6 mg% and IgM 4·2$\pm$2·8 mg%, while in the six coeliac patients they were IgG 7·8$\pm$3·5 mg%, IgA 15·7$\pm$9·2 and IgM 4·7$\pm$2·5 mg%. Although the immunoglobulin content of the intestinal fluid varied between individuals, there appeared to be little difference between the two groups of patients. There was also no apparent

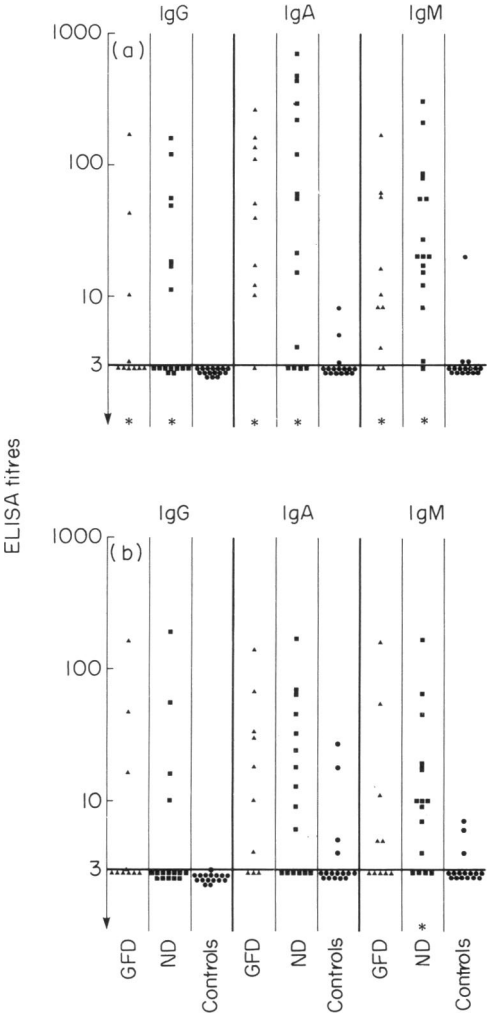

Fig. 1. Intestinal antibody to (a) Frazer's fraction III and (b) beta-lactoglobulin in the IgG, IgA and IgM immunoglobulin classes, measured using an ELISA in intestinal aspirate from coeliac children on a gluten-free diet (GFD), a diet containing gluten (ND) and from controls. $*P < 0{\cdot}05$ for the difference between controls and the group indicated.

correlation between the titre of specific anti-gluten antibody and the concentration of immunoglobulin (data not shown).

Serum antibody to gluten derived antigens. When the diet of the coeliac children contained gluten, the levels of gluten-antibody in their serum were markedly elevated compared to those found in control subjects (Fig. 2, $P < 0{\cdot}001$ for IgG, IgA and IgM antibody to Frazer's fraction III). However, serum antibodies to the non-wheat antigen (β-LG) in these patients, were comparable to those in controls (Fig. 2).

A striking decline in the level of these gluten-specific antibodies (particularly of the IgA class) was seen when the coeliac children transferred to a gluten-free diet (Figs 2 & 3). The results shown in Fig. 3 clearly demonstrate that when the coeliac children are on a gluten-free diet, the titre of serum antibody to the gluten-derived antigen, Frazer's fraction III, approaches those found in normal subjects. This drop in serum antibody to gluten antigens contrasted with the persistent elevation of intestinal antibody to these antigens (Fig. 3). Lastly, there was no significant change in the level of serum antibody to the other food proteins after the exclusion of dietary gluten (Figs 2 & 3).

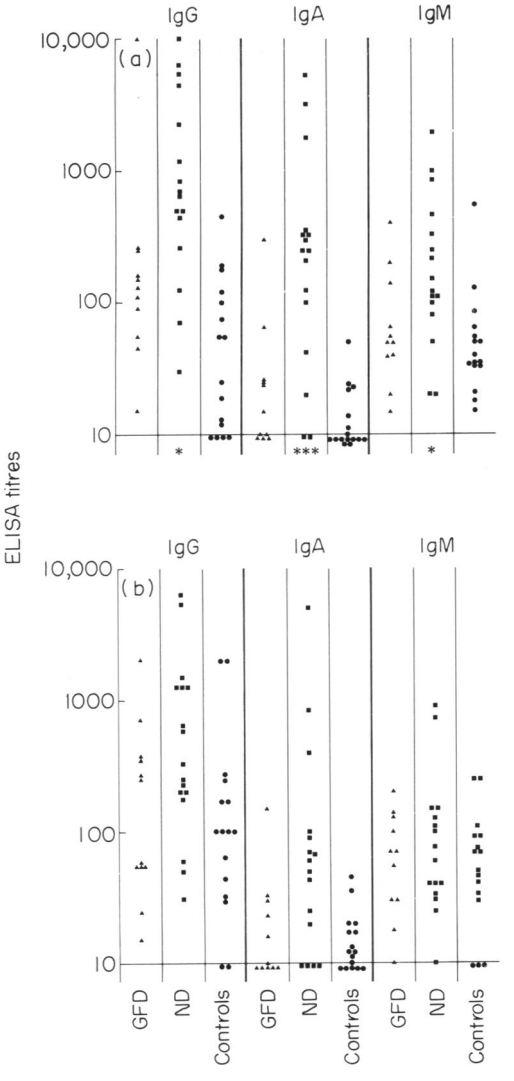

Fig. 2. Serum antibody to (a) Frazer's fraction III and (b) beta-lactoglobulin in the IgG, IgA and IgM immunoglobulin classes, measured using an ELISA in samples from coeliac children on a gluten-free diet (GFD), a diet containing gluten (ND) and from controls. $*P < 0{\cdot}05$ for the difference between coeliacs on a diet containing gluten and controls. $***P < 0{\cdot}05$ for the difference between coeliacs on a diet containing gluten and both coeliacs on a gluten-free diet and controls.

DISCUSSION

The marked specialization of the immune system in the gut is manifested by the secretion of sIgA into the intestinal lumen, the specialized populations of T-lymphocytes in the gut (Elson, Heck & Strober, 1979) and the organization of the lymphoid system in the intestine (Parrott, 1976). This makes measurements of serum antibody or the degree of sensitization of peripheral blood lymphocytes a poor substitute for directly measuring immunity at the local level. Our previous studies of bacterial gastroenteritis emphasized strongly the marked degree of dissociation that occurs between antibody responses in serum and at the local site of disease (LaBrooy *et al.*, 1980). They also demonstrated that the more modern assay techniques, such as solid phase radioimmu-

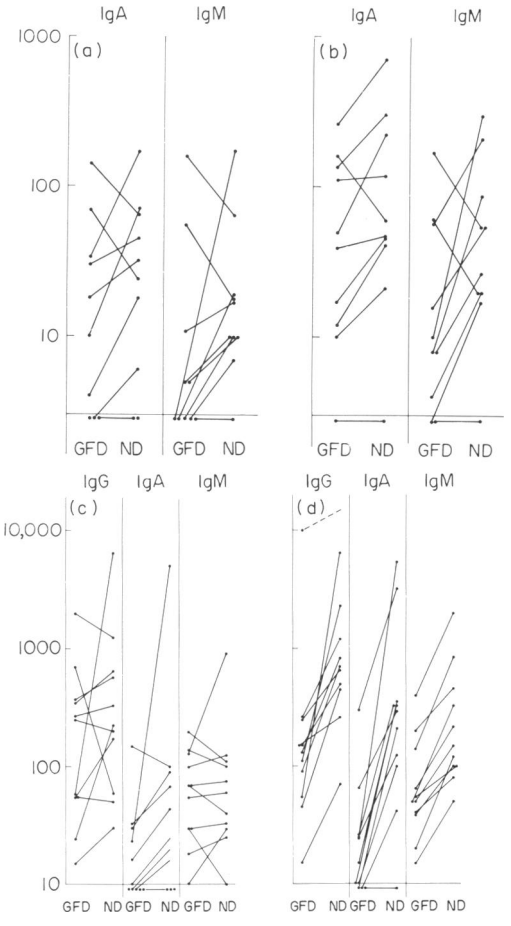

Fig. 3. Serial changes in ELISA titres of (a, b) intestinal and (c, d) serum antibody in the IgG, IgA and IgM classes to (b, d) a gluten antigen (Frazer's fraction III) and (a, c) another food-protein (β-lactoglobulin) in 10 coeliac children sampled while taking (ND) and avoiding (GFD) dietary gluten.

noassays and enzyme-linked immunoabsorbent assays, are able to measure intestinal antibody with greater accuracy than the older assays such as precipitation in agarose-gels, bacterial agglutination or haemagglutination. These reasons prompted us to re-examine the antibody response in serum and gut-secretions in coeliac disease.

The most striking difference between the specific antibody response in the intestine compared to that in serum was its relative persistence in children on a gluten-free diet, at a time when their serum response had returned to near control levels. This pattern is similar to our previous observations of the immune response to bacterial infection. Although a marked response was seen in both serum and secretions shortly after infection, there was evidence of heightened immunity at the local level one year after serum antibody had declined (LaBrooy, Shearman & Rowley, 1982). Whether the persistence of anti-gluten antibody in the intestine of coeliacs reflects continued intake of minute amounts of gluten in the diet or merely much greater initial priming at this level compared to that of the systemic compartment, is an unanswered question. All the intestinal samples collected when the coeliac patients were on a gluten-free diet were taken at a time when the mucosal histology had clearly improved, although some patients still showed a mild degree of partial villous atrophy. Whether raised intestinal antibody levels persist years after subjects transfer to a gluten-free diet and their intestinal histology has reverted completely to normal, remains to be studied.

One of the reasons for examining the class of anti-gluten antibody present, was the possibility of recognizing a measure that would allow a clear cut diagnosis of coeliac disease to be made. While the concentrations of anti-gluten antibody in the coeliac group was much higher than in the control group, particularly in the intestinal secretions, there was some overlap between the groups with antibody measurable in a few of the controls and absent in some of the patients. This necessitates the conclusion that no single test could be put forward as an alternative to intestinal biopsy for the diagnosis of coeliac disease. The decline in serum antibody to the gluten-derived antigens, particularly of the IgA class, in patients on a gluten-free diet suggests the possibility that its serial measurement may be of value in assessing the effectiveness of gluten withdrawal and patient-compliance with dietary instructions. The concentrations of antibody to the other protein antigens reinforced previous findings of others (Ferguson & Carswell, 1972). Coeliac children tended to have higher levels of antibody to these antigens than did the control subjects, though the difference between the two groups was no where near as striking as with the antigens derived from gluten. One of the explanations advanced for the frequency of antibodies to food proteins in the serum and secretions of patients with coeliac disease is the increased permeability of the intestinal mucosa during the active stage of the disease. In this study there was no consistent change in antibody levels to BSA or β-LG, in spite of the marked improvement in the intestinal lesion when children were put on a gluten-free diet. This could conceivably result from persistence of increased permeability of the intestinal mucosa in coeliac disease even while patients are in clinical and histological remission, as described recently by Bjarnason, Peters & Veall (1983). Another possibility is that an increased immune reactivity to oral antigen is an intrinsic feature of coeliac disease. Either explanation could be of relevance to the development of coeliac disease, as gluten has the characteristic of relative indigestibility (Cornell & Townley, 1973) and consequently possesses a greater potential than other food proteins for being absorbed in an antigenic form and trigger an immune response in the wall of the intestine.

This study emphasizes that immunological reactivity to gluten, which is a well described feature of coeliac disease, is more clearly apparent in antibody present in gut secretions than in the serum. Our findings also underline the fact that the increase in immune reactivity to gluten antigens is much greater than that seen to other food proteins in coeliac disease. While no single measure in this study would discriminate between children with coeliac disease and the non-coeliac control group, the findings are compatible with the hypothesis that immunological hyper-reactivity to gluten, at the level of the intestine in coeliac patients, plays a central role in the development of coeliac disease.

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REFERENCES

BJARNASON, I., PETERS, T.J. & VEALL, N. (1983) A persistent defect in intestinal permeability in coeliac disease demonstrated by a ^{51}Cr-labelled EDTA absorption test. *Lancet* **i**, 323.

BULLEN, A.W. & LOSOWSKY, M.S. (1976) Cell-mediated immunity to gluten fraction III (GF III) in adult coeliac disease. *Gut* **17**, 813.

BURGIN-WOLFF, A., BERTELE, R.M., BERGER, R., GAZE, H., HARMS, H.K., JUST, M., KHANNE, S., SCHRIMM, K., SIGNER, E. & TOMOVIC, D. (1983) A reliable screening test for childhood coeliac disease. Fluorescent immunosorbent test for gliadin antibodies. *J. Pediatrics* **102**, 655.

CORNELL, H.H. & TOWNLEY, R.R.W. (1973) Investigation of possible intestinal peptidase deficiency in coeliac disease. *Clinica chim. Acta* **43**, 113.

DAVIDSON, M. & WASSERMAN, R. (1966) The irritable colon of childhood (chronic non-specific diarrhoea syndrome). *J. Pediatrics* **69**, 1027.

ELSON, C.O., HECK, J.A. & STROBER, W. (1979) T-cell regulation of murine IgA synthesis. *J. exp. Med.* **149**, 632.

FALCHUK, Z.M. & STROBER, W. (1974) Gluten-sensitive enteropathy: synthesis of anti-gliadin antibody in vitro. *Gut* **15**, 947.

FERGUSON, A. & CARSWELL, F. (1972) Precipitins to dietary proteins in serum and upper intestinal secretions of coeliac children. *Brit. med. J.* **i**, 75.

FIRER, M.A., HOSKING, C.S. & HILL, D.J. (1981) Effect of antigen load on development of milk antibodies in infants allergic to milk. *Brit. med. J.* **283**, 693.

FORD, R.P.K. (1981) A simple method of duodenal juice collection in association with small bowel biopsy in children. *Aust. Paed. J.* **17,** 54.

FRAZER, A.C., FLETCHER, R.F., ROSS, C.A.C., SHAW, B., SAMMONS, H.G. & SCHNEIDER, R. (1959) Gluten induced enteropathy. The effect of partially digested gluten. *Lancet* **ii,** 252.

HERSKOVIC, T., KATZ, J., GRYBOWSKI, J.D. & SPIRO, H.M. (1968) Coproantibodies to gluten in coeliac disease. *J. Amer. Med. Assoc.* **203,** 877.

HOHMANN, A., LABROOY, J., DAVIDSON, G.P. & SHEARMAN, D.J.C. (1983) Measurement of specific antibodies in human intestinal aspirate: effect of the protease inhibitor phenylmethyl-sulphonyl fluoride. *J. immunol. Methods*, **64,** 199.

KATZ, J., KANTOR, F.S. & HERSKOVIC, T. (1968) Intestinal antibodies to wheat fractions in coeliac disease. *Ann. int. Med.* **69,** 1149.

LABROOY, J.T., DAVIDSON, G.P., SHEARMAN, D.J.C. & ROWLEY, D. (1980) The antibody response to bacterial gastroenteritis in serum and secretions. *Clin. exp. Immunol.* **41,** 290.

LABROOY, J.T., SHEARMAN, D.J.C. & ROWLEY, D. (1982) Antibodies in serum and secretions 1 year after Salmonella gastroenteritis. *Clin. exp. Immunol.* **48,** 551.

LOEB, P.M., STROBER, W., FALCHUK, Z.M. & LASTER, L. (1972) Incorporation of leucine-^{14}C into immunoglobulins by jejunal biopsies of patients with coeliac sprue and other gastrointestinal diseases. *J. clin. Invest.* **50,** 559.

MCNEISH, A.S., HARMS, H.K., REY, J., SCHMERLING, D.H., VISAKORPI, J.K. & WALKER-SMITH, J.A. (1979) The diagnosis of coeliac disease. Commentary on the current practices of members of the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN). *Arch. Dis. Child*, **54,** 783.

O'FARRELLY, C.O., FEIGHERY, C., GREALLY, J.F. & WEIR, D.G. (1982) Cellular response to alpha-gliadin in untreated coeliac disease. *Gut* **23,** 83.

O'FARRELLY, C., KELLY, J., HEKKENS, W., BRADLEY, B., THOMPSON, A., FEIGHERY, C. & WEIR, D.G. (1983) α-Gliadin antibody levels: a serological test for coeliac disease. *Br. med. J.* **286,** 2007.

PARROTT, D.M.V. (1976) The gut as a lymphoid organ. *Clinics in Gastroenterology* **5,** 211.

SAVILAHTI, E., PERKKIO, M., KALIMO, K., VIANDER, M., VAINIO, E. & REUNALA, T. (1983) IgA antigliadin antibodies: a marker of mucosal damage in childhood coeliac disease. *Lancet* **i,** 320.

UNSWORTH, D.J., KIEFFER, M., HOLBORROW, E. COOMBS, R.R.A. & WALKER-SMITH, J. (1981) IgA antigliadin antibodies in coeliac disease. *Clin. exp. Immunol.* **46,** 286.

WEISS, J.B., AUSTIN, R.K., SCHANFIELD, M.S. & KAGNOFF, M.F. (1983) Gluten sensitive enteropathy. Immunoglobulin G heavy-chain (Gm) allotypes and the immune response to wheat gliadin. *J. clin. Invest.* **72,** 96.