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# Serum IgG subclass antibodies to gliadin and other dietary antigens in children with coeliac disease

S. HUSBY,*† N. FOGED,† V.-A. OXELIUS‡ & S-E. SVEHAG* **Institute of Medical Microbiology, Odense University, Denmark †Department of Pediatrics, Odense University Hospital, Denmark and ‡Department of Pediatrics, Lund University Hospital, Sweden*

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## SUMMARY

IgG subclasses of antibodies to the dietary antigens gliadin, glycgli (a gluten component), ovalbumin (OA) and $\beta$-lactoglobulin (BLG) were quantified in children with coeliac disease (CD), nine on a gluten-containing diet, 15 on a gluten-free diet, and in appropriate controls. In addition, total serum IgG subclasses were measured.

IgG1 and IgG3 antibodies to gluten and glycgli were detected in 9/9 and 8/9 CD-patient on a gluten-containing diet, respectively, and in 4/15 and 6/15 patients on a gluten-free diet. None of the controls had appreciable levels of IgG1 antibodies and only 1/22 of the controls had IgG3 antibodies to gliadin and glycgli. IgG2 and IgG4 antibodies to the same antigens were found in a few coeliacs and controls. Consecutive samples from coeliac children (8 patients) showed a clear relation between the exposure to gluten and a rise in IgG1 (8/8) and IgG3 antibody levels (7/8).

In contrast, IgG antibodies to OA and BLG were almost exclusively of the IgG1 and IgG4 subclasses. The highest levels were found in children with CD, but the differences between the groups were not significant. Total serum IgG subclasses did not differ between the groups, but the IgG2 and IgG4 levels in most coeliac children were low. The production of IgG1 and IgG3 antibodies to gluten components may be an important precondition for the development of coeliac disease in susceptible individuals.

**Keywords** IgG subclass antibodies gliadin dietary antigens coeliac disease children

## INTRODUCTION

Coeliac disease is caused by the ingestion of gluten that in susceptible individuals causes damage to the small intestinal mucosa. The pathogenic mechanisms have not been definitely clarified, although immunological factors have been implicated (Strober, 1975). A large proportion (70–90%) of coeliac patients possess the HLA-DR3 or DR7 haplotypes (Svejgaard & Ryder, 1977; Pena *et al.*, 1978) and patients lacking these haplotypes have preferentially the immunoglobulin heavy chain $G_m$ allotype f, n, b (Kagnoff *et al.*, 1983).

Raised serum antibody titres against gluten or its pathogenically active component gliadin have been found in coeliac disease (Kenrick & Walker-Smith, 1970; Cornell, 1974; Eterman *et al.*, 1977). The antibodies were mainly of the IgG and IgA classes. The presence of anti-gliadin or anti-reticulin antibodies of the IgA-class were considered almost 100% diagnostic for coeliac disease (Savilahti *et al.*, 1983; Unsworth *et al.*, 1981; Scott *et al.*, 1984, Stenhammer *et al.*, 1984). However, other

Correspondence: Dr S. Husby, Institute of Medical Microbiology, Odense University, J. B. Winslows Vej 19, DK-500 Odense C, Denmark.

investigations have shown anti-gliadin and anti-reticulum antibodies in up to one third of children with inflammatory bowel disease (Unsworth *et al.*, 1983; 1985).

The aim of the present study was to investigate the levels of serum IgG subclasses and the IgG subclass distribution of serum antibodies to gluten products and to two other dietary antigens in children with coeliac disease as compared with appropriate controls.

## MATERIALS AND METHODS

*Patient sera.* Coeliac disease was diagnosed in a total of 15 children according to the diagnostic criteria recommended by the European Society for Paediatric Gastroenterology and Nutrition (McNeish *et al.*, 1979). Serum samples were obtained from 15 patients on a gluten-free diet for at least 1 year (age 2–15 years, median 7 years). In eight of the children additional blood samples were taken at gluten-exposure. The consecutive serum samples were taken at the primary admission (1. jejunal biopsy) and after 1–2 years of diet (2. biopsy, four children), and/or at the 2. biopsy and after gluten-provocation (minimum of 10 g gluten per day) for 3 months or at gastrointestinal symptoms (3. biopsy, six children). One patient had a blood sample taken at gluten provocation only. Serum from one patient at initial admission was only available in sufficient amounts to test for the presence of anti-gliadin and anti-glycgli antibodies and total IgG subclasses, but not for anti-OA and anti-BLG antibodies.

*Control sera.* Serum samples were obtained from 10 children (age $1\frac{1}{2}$–$12\frac{1}{2}$ years, median $4\frac{1}{2}$ years), who at the clinical suspicion of coeliac disease had undergone a jejunal biopsy, which was found normal (disease controls). Furthermore, serum was obtained from 12 children, also with approximately the same age distribution (age 2–14 years, median 8 years), admitted at the hospital for surgery with no gastrointestinal symptoms or signs and with no infections (normal controls).

*Antigens and antisera.* Ovalbumin (OA, grade V, crystalline), β-lactoglobulin (BLG, crystalline, A+B), gluten and gliadin were purchased from Sigma (St Louis, MO, USA). Human serum albumin (HSA, reinst) was from Behringwerke (Marburg, FRG). A water-soluble digest of gluten, glyc-gli, was prepared according to Douglas (1976). 50 g of wheat gluten (Sigma, St Louis, MO, USA) was suspended in 1·5 l of 0·1 M sodium ethanoate (sodium acetate), 2 M ethanol, pH 6·5, stirred for 12 h and centrifuged (500 *g* for 15 min). To the supernatant was added 250 ml 2·2 M trichloroacetic acid, and the mixture was incubated for 1 h on ice and centrifuged (18000 *g* for 1 h). The precipitate was redissolved in ethanoic (acetic) acid, dialysed and freeze dried.

Human λIgG myelomas of the four subclasses were a gift from WHO (Dr F. Skvaril, Berne, Switzerland).

Normal rabbit $F(ab')_2$-fragment and rabbit $F(ab')_2$ anti-λ human IgG were prepared as previously described (Husby, Jensenius & Svehag, 1985). Monoclonal mouse anti-human IgG subclass antibody was purchased as ascitic fluid from Seward (London, UK). Alkaline phosphatase (AP)-labelled rabbit anti-mouse IgG was from Orion (Helsinki, Finland) and was absorbed with Sepharose 4B bound human IgG before use.

*Enzyme-linked immunosorbent assay (ELISA) for IgG subclasses of antibodies to dietary antigens.* The assays were carried out in principle as previously described for IgG subclass antibodies to OA and BLG (Husby *et al.*, 1985). Microplate wells (Immunoplate II, Nunc, Kamstrup, Denmark) were coated with gliadin, glyc-gli, OA, BLG, or HSA as control, at 9 μg/ml of 0·1 M bicarbonate buffer, pH 9·6. To obtain a quantitative estimate of the IgG subclass binding, one subclass was tested per plate, and separate wells were coated with rabbit $F(ab')_2$ anti-λ antibody and normal rabbit $F(ab')_2$ at 2·5 μg/ml bicarbonate buffer. Non-specific binding was blocked with 0·1% HSA in Tris-buffered 0·15 M NaCl (TBS) for 1 h followed by three times washing in TBS with 0·05% Tween 20 (TBS-Tween). Sera diluted 1 : 100 or 1 : 500 in TBS-Tween were incubated overnight in the antigen-coated wells. IgG myeloma standards, 60–0·8 ng/ml for IgG1, IgG3 and IgG4 and 600–2·0 ng/ml for IgG2, in 1% normal rabbit serum, were incubated overnight in $F(ab')_2$ anti-λ antibody and normal rabbit $F(ab')_2$-coated wells.

The plates were washed and incubated with monoclonal anti-IgG subclass antibody for 4 h, followed by AP-rabbit anti-mouse IgG antibody for 2 h, and development with *P*-nitrophenyl

phosphate at 1 mg/ml of 10% diethanolamine buffer. Absorbance at 405 nm was read on a multichannel spectrophotometer (NJ-2000, Nippon-Intermed, Tokyo, Japan).

From the myeloma standards ($OD_{405}$-values from normal rabbit $F(ab')_2$-coated wells subtracted) the amount of antibody of the individual subclass bound to the antigen-coated wells (HSA-coated wells subtracted) was estimated. The detection limit for the IgG1-assay and for the IgG4-assay was 0·08 μg/ml of undiluted serum, for the IgG2-assay about 2 μg/ml and for the IgG3-assay 0·25 μg/ml. All determinations were done in duplicate.

A selected positive control serum was included in each run ($n=16$) for each subclass and antigen. The within-run coefficients of variation (CV) for anti-gliadin and anti-glycgli antibodies were: in IgG1 4% and 7%, in IgG2 3% and 4%, in IgG3 4% and 21% and in IgG4 4% and 7%, respectively. The between-days CV's for anti-gliadin and anti-glycgli antibodies were: in IgG1 50% and 40%, in IgG2 36% and 25%, in IgG3 44% and 21% and in IgG4 38% and 40%, respectively. For anti-OA and anti-BLG antibodies the within-run CV for IgG1, IgG2 and IgG4 antibodies ranged 4–7% and the between-day CV 25–39%. No positive IgG3 control serum was available for the latter antibodies. Consecutive samples from the same patient and corresponding numbers of patient and control sera were tested in the same run.

*Total IgG subclasses.* Determination of total serum IgG subclasses was performed by radial immunodiffusion with rabbit antisera, raised according to Oxelius (1978). Antisera were rendered specific for IgG by absorption with serum fractions or isolated myeloma proteins. Subclass specificity was secured by absorption with isolated myeloma proteins including all relevant heavy-light chain combinations and IgG subclasses with different Gm markers. Also IgG-deficient sera were used.

*Statistics.* For comparison between groups was used the non-parametric Mann-Whitney U-test for unpaired samples, as a two-tailed test. The level of significance was chosen as $P=0{\cdot}05$.

## RESULTS

### *IgG subclasses of anti-gliadin and anti-glycgli antibodies*

Anti-gliadin antibody and anti-glycgli antibody in general followed each other closely. IgG1 antibodies (Fig. 1) were regularly high in coeliac patients on a gluten-containing diet (median 6·7 μg/ml, range 3·6 – > 33·4 μg/ml, and median 9·8 μg/ml, range 2·85 – > 33·4 μg/ml), respectively and present in lower concentrations in 4/15 coeliac children on a gluten-free diet (median 0·34 μg/ml, range 0–15·5 μg/ml, and median 0·55 μg/ml, range 0–18·5 μg/ml). Three out of 10 disease control sera and 2/12 normal controls had low levels of IgG1 antibody. IgG2 antibodies were observed in 4/9 coeliacs on a normal diet and in 4/15 on a gluten-free diet, in 6/10 disease controls and in a single normal control. IgG3 antibodies were detected in all but one coeliac patients exposed to gluten (median 2·4 μg/ml, range 0–6·5 μg/ml, and median 2·1 μg/ml, range 0–5·6 μg/ml), in 6/15 patients on a gluten-free diet (median 0, range 0–5·5 μg/ml, and median 0, range 0–5·5 μg/ml), in one disease control and in no normal controls. Low levels of IgG4 antibodies were discovered in a few children in all groups.

The levels of IgG1 antibodies to glycgli and gliadin were higher in coeliac children on a normal diet than in each of the other groups ($P<0{\cdot}001$). IgG3 anti-glycgli and anti-gliadin antibodies in coeliac children on a normal diet were also significantly higher than in patients on a gluten-free diet ($P<0{\cdot}004$ and $P<0{\cdot}011$, respectively), in disease controls ($P<0{\cdot}006$) and in normal controls ($P<0{\cdot}001$). IgG2 and IgG4 anti-glycgli and anti-gliadin antibodies were not significantly different in the groups studied ($P>0{\cdot}05$) except IgG2 antibodies to glycgli, which in coeliac patients on a normal diet barely differed to the normal controls ($P<0{\cdot}045$). Thus, the IgG1 and IgG3 antibodies discriminated rather effectively between the coeliac children and the controls. However, out of 10 sera from apparently healthy adults, one serum with appreciably high IgG1 and IgG3 anti-gliadin antibodies was discovered and used as a performance control serum in the assays for IgG1 and IgG3 antibodies.

Consecutive measurements of IgG subclass antibodies in eight coeliac children during investigation for coeliac disease (Figs 2 & 3) revealed a marked decrease of anti-gliadin and anti-

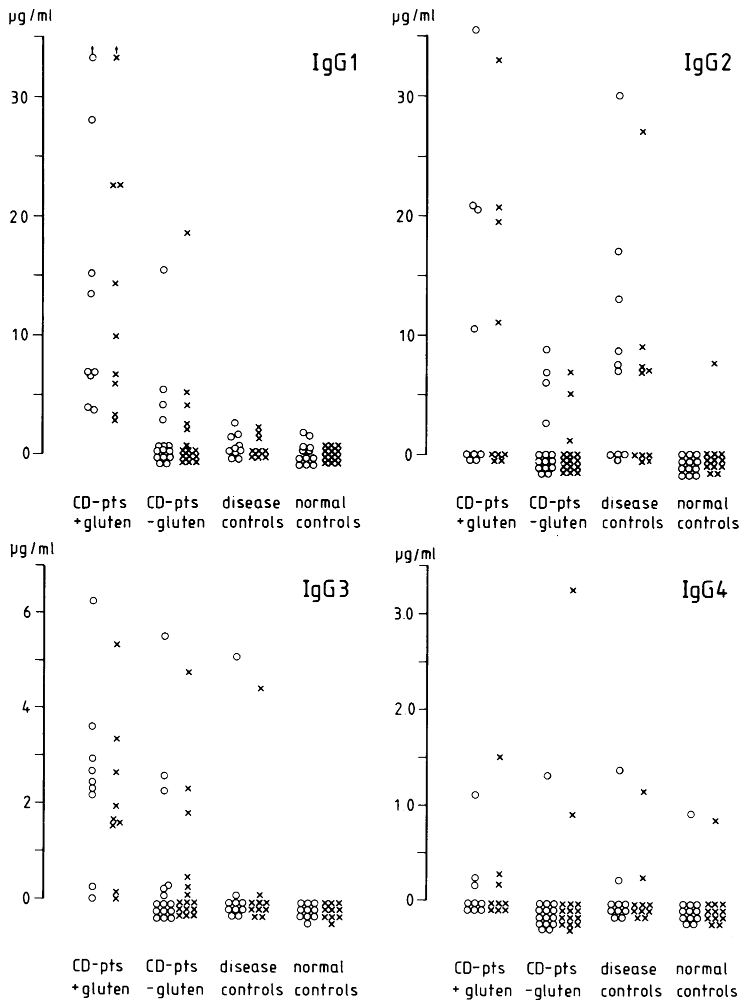


**Fig. 1.** Anti-gliadin (○) and anti-glycgli (×) antibodies of the four IgG subclasses in coeliac children on a gluten containing diet (CD +gluten), in coeliac children on a gluten-free diet (CD −gluten), patients in disease controls and in normal controls.

glycgli IgG1 and IgG3 antibody levels during the gluten-free diet period and a vigorous rise after gluten provocation. However, one patient showed (Figs 2 & 3) increased IgG3 antibody values on a gluten-free diet as compared to the initial admission, followed by a further increase at provocation with gluten. In three patients IgG2 antibodies rose at the exposure to gluten, while the rest were IgG2 antibody negative. Two children showed an increase in IgG4 antibodies, and the rest were antibody negative.

*IgG subclasses of anti-OA and anti-BLG antibodies*

Serum anti-OA and anti-BLG antibodies were detectable almost exclusively in IgG1 and IgG4 (Table 1). Low levels of IgG3 anti-BLG antibodies were detected in one coeliac patient exposed to gluten, one on a gluten-free diet and one disease control. One coeliac child on a gluten-free diet had low IgG2 and IgG3 antibodies.

Coeliac patients tended to have higher IgG1 and IgG4 antibody levels than controls, but the differences were not significant ($P > 0{\cdot}05$). The IgG1 and IgG4 anti-OA antibodies rose in 4/6 cases

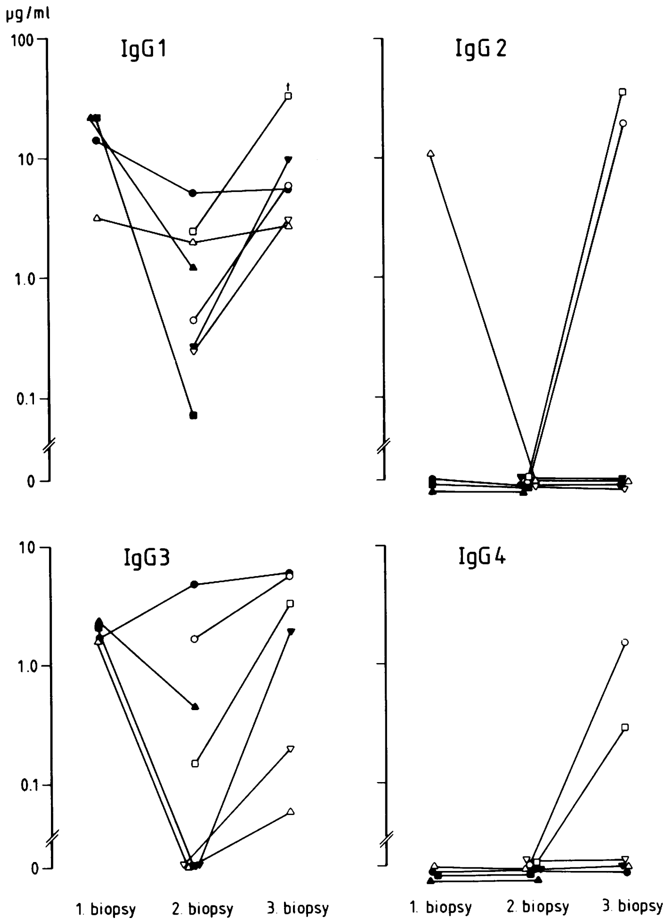


**Fig. 2.** Consecutive measurements of IgG subclass anti-glycgli antibodies (IgG1, IgG2, IgG3 and IgG4) in 8 coeliac children during the diagnostic procedure: at primary admission (1. biopsy), after gluten-free diet for at least 1 year (2. biopsy) and/or after gluten provocation (3. biopsy). Note logarithmic scale of the ordinate.

upon exposure to gluten, whereas IgG1 and IgG4 anti-BLG antibodies rose in 2/6 and 3/6 cases, respectively (data not shown).

*Total IgG subclasses in serum*

The levels of serum IgG subclasses were tested in the coeliac patients and in the disease control. No significant differences were disclosed between the patient groups (Table 2). However, IgG2 and IgG4 levels tended to be low in all three groups as compared to the normal values. Three coeliac children had serum IgG2 levels below the normal range. No trend was apparent in subclass levels in consecutive serum samples from coeliac patients (data not shown).

## DISCUSSION

IgG subclasses of antibodies to wheat gluten fraction B were recently investigated in adult coeliac patients on a normal diet, employing semi-quantitative ELISA's and either polyclonal or monoclonal antisera. Antibodies were found in all four subclasses (Rawcliffe, Jewell & Faux, 1985). In the present study the anti-gliadin and anti-glycgli antibody levels were regularly high in the IgG1 and IgG3 subclasses in coeliac patients on a gluten-containing diet, and virtually absent in disease

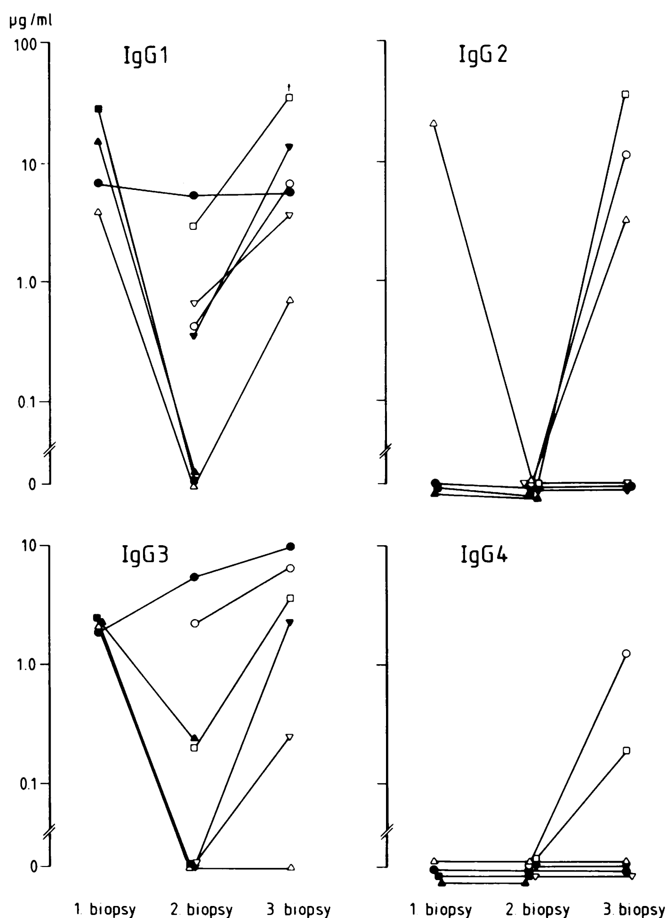


**Fig. 3.** Consecutive measurements of IgG subclass anti-gliadin antibodies in eight coeliac children during the diagnostic procedure: at primary admission (1. biopsy), after gluten-free diet for at least 1 year (2. biopsy) and/or after gluten provocation (3. biopsy).

**Table 1.** Serum IgG subclass antibodies to ovalbumin (OA) and β-lactoglobulin (BLG)

| | Anti-OA antibodies (μg/ml) | | | | Anti-BLG antibodies (μg/ml) | | | |
|---|---|---|---|---|---|---|---|---|
| Patient group | IgG1 | IgG2 | IgG3 | IgG4 | IgG1 | IgG2 | IgG3 | IgG4 |
| Coeliac patients median | 1·1 | 0 | 0 | 2·9 | 0·1 | 0 | 0 | 0·63 |
| (+gluten) $n=8$ (range) | (0–2·7) | (0–0) | (0–0) | (0–19) | (0–3·4) | (0–0) | (0–2·5) | (0–12·5) |
| Coeliac patients median | 0·57 | 0 | 0 | 1·85 | 0·3 | 0 | 0 | 1·45 |
| (−gluten) $n=15$ (range) | (0–2·4) | (0–0) | (0–0) | (0–11·8) | (0–2·5) | (0–13·0) | (0–0·84) | (0–7·8) |
| Disease controls median | 0–0 | 0 | 0 | 0·52 | 0 | 0 | 0 | 2·7 |
| $n=10$ (range) | (0–0·54) | (0–0) | (0–0) | (0–4·1) | (0–40) | (0–0) | (0–1·1) | (0–10·7) |
| Normal controls median | 0 | 0 | 0 | 0·3 | 0 | 0 | 0 | 0 |
| $n=12$ (range) | (0–1·4) | (0–0) | (0–0) | (0–3·5) | (0–12·2) | (0–0) | (0–0) | (0–3·6) |

In all cases the concentration 0 μg/ml denotes below the detection level of the various assays (see Materials and Methods).

**Table 2.** Total IgG subclasses in sera from coeliac children and disease controls. Values calculated as the percentage of the mean concentration in healthy children of the same age (Oxelius, 1979)

| Patient group | | IgG1 (%) | IgG2 (%) | IgG3 (%) | IgG4 (%) |
|---|---|---|---|---|---|
| Coeliac patients | median | 87 | 55 | 86 | <3 |
| (+gluten) $n=9$ | (range) | (33–165) | (28–143) | (64–161) | (<3–116) |
| Coeliac patients | median | 99 | 74 | 110 | 21 |
| (−gluten) $n=15$ | (range) | (55–197) | (30–150) | (69–250) | (<3–182) |
| Disease controls | median | 71 | 59 | 75 | 71 |
| $n=10$ | (range) | (58–95) | (13–97) | (53–286) | (<3–128) |

controls and normal controls. Consecutive measurements of sera from coeliac patients (Figs 2 & 3) on and off a gluten-free diet revealed, for IgG1 and IgG3 antibodies, decreasing antibody levels after a gluten free diet, and a vigorous antibody response with reexposure to gluten. IgG2 antibody levels were not detectable in 5/8 coeliac patients, perhaps due to the high threshold ($\sim$0·5 μg/ml serum) for detection in the IgG2 antibody assays. However, IgG2 antibodies did occur in some control sera (Fig. 1).

The levels of serum IgG subclass antibodies to the gluten components gliadin and glycgli followed each other closely (Figs 1–3). No clear difference in the assay reproducibilities (within-run CV's 3–21%, and between-day CV's 22–50%) were observed using these two antigen preparations. The between-day CV's were high, possibly related to the non-covalent binding of antigen or antibody to the solid-phase or to the multiple washing steps. We have compensated for this by including as many sera as possible and both patient and control sera in each run.

Other investigators (Savilahti *et al.*, 1983; Scott, Ek & Brandtzaeg, 1985) have analysed antibody isotypes to dietary antigens in coeliac disease and reported that serum levels of IgG and IgA antibodies in coeliac children were dependent upon exposure to gluten. These investigators noted that IgG anti-gliadin antibodies declined much more slowly than IgA antibodies or not at all at treatment with a gluten-free diet. We observed a clear relationship between exposure to gluten and the levels of anti-gliadin and anti-glycgli antibodies in IgG1 and IgG3 with the exception of IgG3 in one patient. However, IgG2 antibodies were found more evenly in coeliac children with or without gluten in their diet. Interestingly, in a recent report high serum IgG anti-gliadin antibody levels persisted despite a gluten-free diet only in those adult coeliac patients who carried the IgG2 heavy chain allotype marker G2m(n) (Weiss *et al.*, 1983). This allotype has been related to high levels of total IgG2 (van der Giessen *et al.*, 1975). In particular in the present study high IgG2 antibody levels were not found in patients with high total IgG2 levels (data not shown). It remains to be shown, whether the IgG2 antibody response is directly related to the presence of the heavy chain G2m(n) allotype.

The IgG subclass antibodies to OA and BLG in coeliacs and controls were dominated by IgG1 and IgG4 (Table 1), in accordance with our previous investigations of IgG subclass antibodies to OA and BLG in normal adults (Husby *et al.*, 1985). The IgG1 and IgG4 anti-OA and anti-BLG antibodies increased in some coeliac children upon exposure to gluten (data not shown), albeit not as regularly as the anti-gluten antibodies. This augmented response may reflect an increased gut permeability for macromolecules in untreated coeliac disease, as indicated by use of probes such as polyethylene glycols (Chadwick, Phillips & Hoffman, 1977), sugars (Menzies *et al.*, 1979) and $^{51}$Cr–EDTA (Bjarnason, Peters & Veall, 1983). However, no direct evidence of an increased gut permeability for proteins has been presented (Pittcher-Willmott *et al.*, 1982).

The occurrence of IgG1 and IgG3 antibodies to gluten components in untreated coeliac patients and not in coeliac patients on a gluten-free diet or in all but one control may be of pathogenic importance, as IgG1 and IgG3 react strongly with complement and Fc-receptors (Spiegelberg, 1974; Unkeless, Fleit & Mellman, 1981; Burton, 1985). The difference between the IgG subclass pattern of anti-gluten antibodies (regularly IgG3, almost no IgG4) and anti-OA and anti-BLG

antibodies (regularly IgG4, no IgG3) is pronounced. IgG1 antibodies were observed against all the antigens tested. It may be speculated that the substantial amounts of IgG4 anti-OA and anti-BLG antibodies could modify or block the effector functions of the IgG1 antibodies in the immune complexes formed with OA and BLG. Such a modification would not take place in gliadin anti-gliadin complexes, lacking IgG4 antibody.

A local immune complex reaction has been suggested to occur in the gut mucosa of adult coeliac patients based on an early (4–6 h) local reaction after exposure to gluten (Anand *et al.*, 1981; Bramble *et al.*, 1985). Complement deposition was occasionally found in the mucosa (Anand *et al.*, 1981), but other investigators have failed to confirm this observation (Perrkiö, Savilahti & Kuitunen, 1981). In untreated coeliac disease the gut mucosa contained increased numbers of both IgG, IgA and IgM-producing B cells, the increase in IgG-containing cells being most pronounced (Scott, Baklien & Brandtzaeg, 1978).

Several lines of research have pointed to the importance of the local cellular immune system in the pathogenesis of coeliac disease. The number of gut mucosal mast cells is increased in coeliac disease (Strobel, Bussutil & Ferguson, 1983; Marsh & Hinde, 1985). Experiments in rodents have indicated that mucosal villous atrophy is T cell dependent (McMowat & Ferguson, 1981; Ferguson, McMowat & Strobel, 1983), and T cells have been reported to dominate the lymphocyte population of the gut mucosa in coeliac patients (Selby, Janossy & Jewell, 1981). However, no quantitative differences were found in T cell subsets between coeliac and normal gut mucosa (Selby *et al.*, 1983), although T cell activation seems to occur (Marsh & Maeney, 1983, Malizia *et al.*, 1985). A systemic T cell mediated hyporesponsiveness to gluten in HLA-DR3 and HLA-DR7 positive coeliac patients was suggested recently (Scott *et al.*, 1983; O'Farelly *et al.*, 1984). However, this suppression may, considering the assay conditions used, be antigen non-specific (Sollid, Scott & Thorsby, 1985).

Our understanding of the pathogenesis of coeliac disease is still meager. Both the humoral and the cellular arms of the immune system seem to be specifically activated. The presentation of gliadin components in combination with certain MHC class II antigens may induce highly efficient local immune responses. Alternatively, a postulated reduced T helper cell function for the production of the IgA isotype may lead to increased antigen absorption and thereby also an increased stimulation of the immune system. In any event, the production of IgG1 and IgG3 anti-gliadin antibodies, as shown in the present study, may upon antigen exposure evoke complement fixation and cellular activation resulting in damage of the gut mucosa.

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