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Alimentary tract and pancreas

Passage of dietary antigens into the blood of children with coeliac disease. Quantification and size distribution of absorbed antigens

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SUMMARY The uptake of ovalbumin (OA) from egg and beta-lactoglobulin (BLG) from cow's milk into the blood was investigated for seven hours after a test meal in five children with coeliac disease on a gluten free diet and after gluten challenge, and in five children with normal jejunal mucosa. Ovalbumin was detectable by ELISA in three of five coeliac children (maximal concentrations 8–178 ng/ml serum) and in five of five controls (maximal 4–91 ng/ml serum). Beta-lactoglobulin was detected in three of five coeliac children (maximal 0·6–6 ng/ml serum) and in two of five controls (maximal 0·5 and 50 ng/ml serum). No clear relationship was seen between maximal antigen concentrations and titres of serum IgG or IgA antibodies determined by ELISA, or as percentage antigen binding in a Farr type radioimmunoassay. Ovalbumin and beta-lactoglobulin was seen in serum of all coeliac patients and controls by HPLC fractionation in combination with ELISA, either in high MW fractions, or at the M_r of native OA and BLG, respectively. In one control degradation products (about 17 kD) of BLG were detectable in serum. The serum concentrations of OA and BLG were increased on gluten challenge in four of five coeliac children, indicating increased macromolecular passage through the gut mucosa in untreated coeliac disease.

The passage of dietary antigens into the blood of healthy adults has previously been demonstrated in minute amounts, probably representing a normal physiological process.[1-3] The uptake of larger amounts of dietary antigens was indicated in normal infants,[4] in children recovering from acute diarrhoea,[5] in atopic children,[6,7] and in hypogammaglobulinaemic[8] or IgA-deficient adults.[9]

Children with coeliac disease have increased titres of serum antibodies to dietary antigens, as compared with normal children,[10-12] possibly because of augmented penetration of dietary antigens from the gastrointestinal tract. Increased gut permeability has been demonstrated in adult coeliac patients by the use of probes such as sugars[13] and ^{51}Cr-EDTA.[14] In contrast, polyethylene glycols were absorbed in decreased amounts as compared with healthy subjects.[15,16] No clear evidence for altered gut permeability for proteins was obtained when the uptake of protein antigen was investigated in six coeliac children.[17]

To further address the question of macromolecular passage across the gut mucosa in patients with coeliac disease we have studied the uptake of two dietary protein antigens in coeliac children on a gluten free diet and after gluten challenge. In addition, the antigen uptake was investigated in children suspected of having coeliac disease, but with the subsequent finding of normal jejunal biopsies. ELISA techniques were used for antigen determination. The absorbed proteins were analysed with regard to their size distribution by high pressure liquid gel permeation chromatography (HPLC) in combination with ELISA, and the levels of the corresponding antibodies were determined.

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Methods

Five children (age 2½–13 years) were studied with a diagnosis of childhood coeliac disease, established by repetitive jejunal biopsies, as recommended by the European Society for Paediatric Gastroenterology and Nutrition.[18] The biopsies were taken under general anaesthesia, with a Quinton pediatric capsule under radiological control at the ligament of Treitz. The procedure was carried out at initial admission, after one year or more on a gluten free diet and if the mucosa was normalised after three months of gluten challenge (minimum 10 g of gluten per day) or on the appearance of severe symptoms. The biopsy was considered completely normal in four children and in one child (no 1) the mucosa was not completely normalised after one year of gluten free diet (Table 1). One of the coeliac patients (no 2) in addition was IgA-deficient.

A gastric tube was installed at the second and third biopsy procedure. One hour after the end of anaesthesia the child was given a test meal consisting of 2 ml raw egg and 10 ml cow's milk per kg body weight through the gastric tube. Blood samples were taken from an indwelling cannula before the test meal (t_0) and 30, 60, 120, 180, 240, and 420 minutes after the meal, during which time the patient took nothing by mouth. Serum was prepared in aliquots and stored at −60°C. The subjects had fasted for at least eight hours before instillation of the test meal.

The disease controls included five children (age 3½–13 years) suspected of coeliac disease and without clinical allergy to milk or egg. These children underwent a jejunal biopsy, which was found normal (Table 2). The experimental procedure was the same as outlined above. The investigation was approved by the Regional Scientific Ethical Committee, and informed consent was obtained.

Table 2 *Clinical data and histology of the jenunal biopsies for five children, investigated for coeliac disease (disease controls)*

No	*Age*	*Sex*	*Biopsy*	*Final diagnosis*
1	3½	♀	Normal	Malnutrition
2	4	♀	Normal	Recurrent enteritis
3	11	♂	Normal	Chronic obstipation
4	4	♀	Normal	Recurrent gastroenteritis
5	13	♂	Normal (slight oedema)	Chronic benign bullous dermatitis of childhood

ELISA FOR THE DETECTION OF OVALBUMIN (OA) AND BETA-LACTOGLOBULIN (BLG) IN HUMAN SERUM

These methods have been described in detail previously.[3] The wells of microplates were coated with affinity purified rabbit F(ab')$_2$ anti-OA (400 ng/ml) or F(ab')$_2$ anti-BLG antibody (1000 ng/ml), raised and purified by us. Control wells were coated with normal rabbit F(ab')$_2$. The wells were quenched with HSA in Tris-buffered saline (TBS) for one hour, followed by washing three times with 0·05% Tween 20 in TBS (TBS-Tween). Test sera or standards diluted 1:10 in TBS-Tween were added and incubated overnight. The wells were washed as above and incubated with biotinylated anti-OA antibody or biotinylated anti-BLG antibody overnight. Development was done with avidin beta-galactosidase for two hours, followed by o-nitrophenyl-beta-D-galactopyranoside for one hour as substrate. All samples were tested in duplicate.

Quantification was obtained by the construction of a calibration curve (0·04-270 ng/ml of OA or BLG). The detection limit of the assays was approximately 0·3 ng antigen/ml serum. Separate serum control preparations for OA-detection showed a between-series coefficient of variation (CV) (n=20) of 0·22 at

Table 1 *Histological characteristics of the jejunal biopsies taken to establish the diagnosis in the five coeliac children. Uptake of dietary antigens was investigated at biopsy (2) and (3)*

Patient no	*Age (yrs)*	*Sex*	*Biopsy (1)*	*Biopsy after diet (2)*	*Biopsy after challenge (3)*
1	4½	♂	Total villous atrophy	Slight crypt hyperplasia, stunted villi	Total villous atrophy, crypt hyperplasia
2*	4½	♂	Total villous atrophy, moderate crypt hyperplasia	Normal	Total villous atrophy, crypt hyperplasia
3	2½	♂	Total villous atrophy	Normal	Total villous atrophy, crypt hyperplasia
4	3½	♂	Total villous atrophy, crypt hyperplasia	Normal	Total villous atrophy, crypt hyperplasia
5	13	♀	Total villous atrophy, crypt hyperplasia	Normal	Partial villous atrophy, crypt hyperplasia

*IgA-deficient

10 ng/ml and 0·37 at 1 ng/ml. The within-series coefficient variation was 0·10 at 10 ng/ml and 0·11 at 1 ng/ml, respectively. The mean duplicate variation for each individual taking part in the study, with detectable OA in serum ranged between 0·01–0·08. For the BLG assay (n=20) the between-series CV was 0·13 at 10 ng/ml and 0·29 at 1 ng/ml, and the within-series CV was 0·03 and 0·07, respectively. The mean duplicate variation for each individual with detectable BLG in serum ranged between 0·03–0·19. This variation was zero both in the OA and BLG assays for individuals, without detectable amounts of antigen in serum.

HIGH PRESSURE LIQUID GEL PERMEATION CHROMATOGRAPHY IN COMBINATION WITH ELISA FOR OA- AND BLG-DETECTION (HPLC-ELISA):
High performance liquid chromatography was carried out as previously described.[19 3] Serum samples were injected with a Waters sample processor. The samples were pumped through a precolumn and a 600×7·5 mm TSK G 3000 SW size separation column (Toyo Soda, Tokyo, Japan), at a flowrate of 0·5 ml/min, in sulphate phosphate buffer. The separation characteristics of the column were determined by the elution volumes of purified protein markers: thyroglobulin (Tg, M_r 6·6×10^5) in the void volume (V_o), IgG (M_r 1·5×10^5), and myoglobin (Mg, M_r 1·7×10^4). The CV of the V_e for the markers were below 0·02 (n=5).

Microplate wells were coated with $F(ab')_2$ anti-OA or $F(ab')_2$ anti-BLG antibody, quenched with HSA and washed three times in TBS-Tween. Twenty five microlitres of 1% Tween 20 in TBS was added to further reduce non-specific binding and fractions from the HPLC column were collected into the microplate. The fractions were analysed for OA and BLG as described above, with a serum calibration curve and control serum samples included in the lower two rows of the plate, as described for ELISA's for antigen detection.

Sera from t_0 of the experiment and the samples with the maximal amounts of OA or BLG (120–420 min samples) were analysed. When no antigen was detected in the unfractionated serum samples by ELISA, the 180 minute sample was chosen for fractionation.

ANTIBODY DETERMINATIONS
A modified antigen binding (Farr type) radioimmuno-assay[20 21] was used to determine serum antibodies to OA and BLG. Serum (1:2, 1:10, 1:50) was mixed with ^{125}I-OA or ^{125}I-BLG, and incubated at 37°C for one hour and overnight at 4°C. Polyethylene glycol 6000 (final concentration 15% in TBS-Tween) was added and the preparation incubated on ice for 30 minutes. After centrifugation and washing, the radioactivity of the precipitate was determined. Antibody titres were expressed as reciprocals of the serum dilution precipitating 10% of the total protein bound radioactivity (the amount precipitated in 10% cold trichloroacetic acid).

Biotin-avidin amplified ELISA was used for the determination of serum anti-OA and anti-BLG antibodies of the IgG[22] and IgA class. Ovalbumin or BLG was coated onto microplates followed by blocking with HSA. Serum samples for IgG antibody determination were diluted 1:100 and 1:500, and for IgA determination 1:50, 1:100 and 1:200, and incubated in the microplate. After washings biotinylated anti-IgG or anti-IgA antibody was added. Development with avidin-beta-galactosidase was done as described for antigen determination. Levels of IgG antibodies were related to dilutions of a serum with high antibody titre and expressed in mU/ml. Titres of IgA antibodies were expressed as the highest dilutions giving a signal significantly above the background.

Results

DETERMINATION OF OVALBUMIN IN SERA
Ovalbumin was detectable in the sera of four of five coeliac patients (Fig. 1). The amounts of OA in serum were in three of four patients clearly higher (2·6–180 ng/ml) at the time of challenge with gluten, when the jejunal mucosa showed villous atrophy, than after one year on a gluten free diet, when the jejunal mucosa was normal. In one patient (no 1), who did not have a fully normalised jejunal mucosa after the diet period (Table 1), slightly larger amounts of OA were seen after the diet period. Ovalbumin was already detectable in the 30 minute samples in three individuals (nos 1, 2, and 5), and at 120 minutes in one case (no 3). A well defined peak of serum OA was seen only in two individuals (nos 1 and 2).

The children, who had a normal jejunal biopsy (disease controls) absorbed varying amounts of OA (0–100 ng/ml) after the test meal, within the same range of magnitude as the coeliac children (Fig. 1, controls). Ovalbumin reached maximal levels 120–240 minutes after the test meal.

DETERMINATION OF BETA-LACTOGLOBULIN IN SERA
Beta-lactoglobulin was observed in the sera of three of five coeliac patients (Fig. 2), but at lower levels than OA (less than 28 ng/ml serum). After a gluten free diet only one individual (no 5) had a detectable level of BLG. This child had measurable amounts of

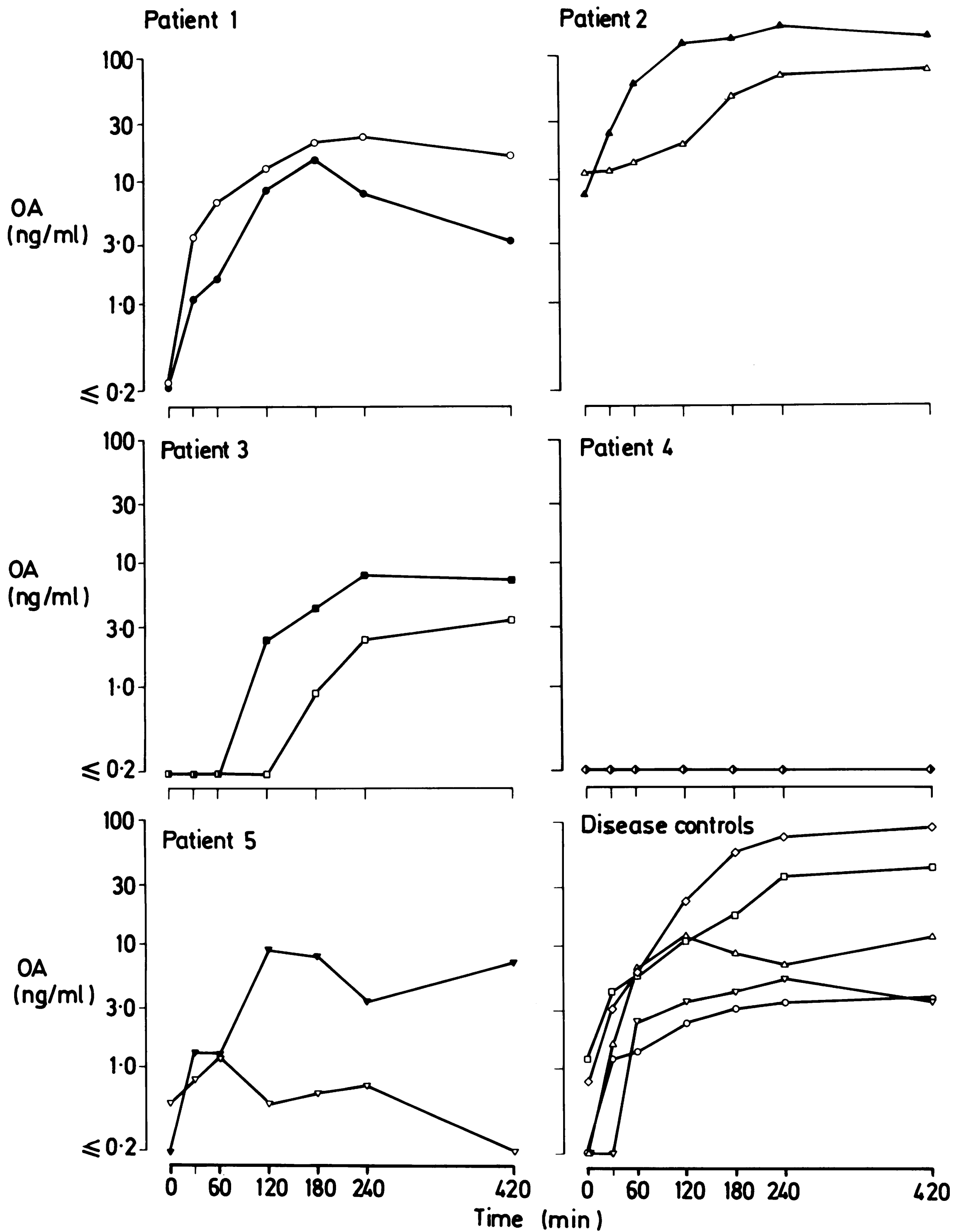

Fig. 1 *Uptake of ovalbumin (OA) after a test meal in five children with coeliac disease on a gluten free diet (open symbols) and after gluten challenge (closed symbols) and in five disease controls. Patient numbers (nos 1–5) are denoted in the Figure. Coeliac patient no 4 did not have detectable OA levels in serum after the test meal. Note logarithmic scale of the ordinate.*

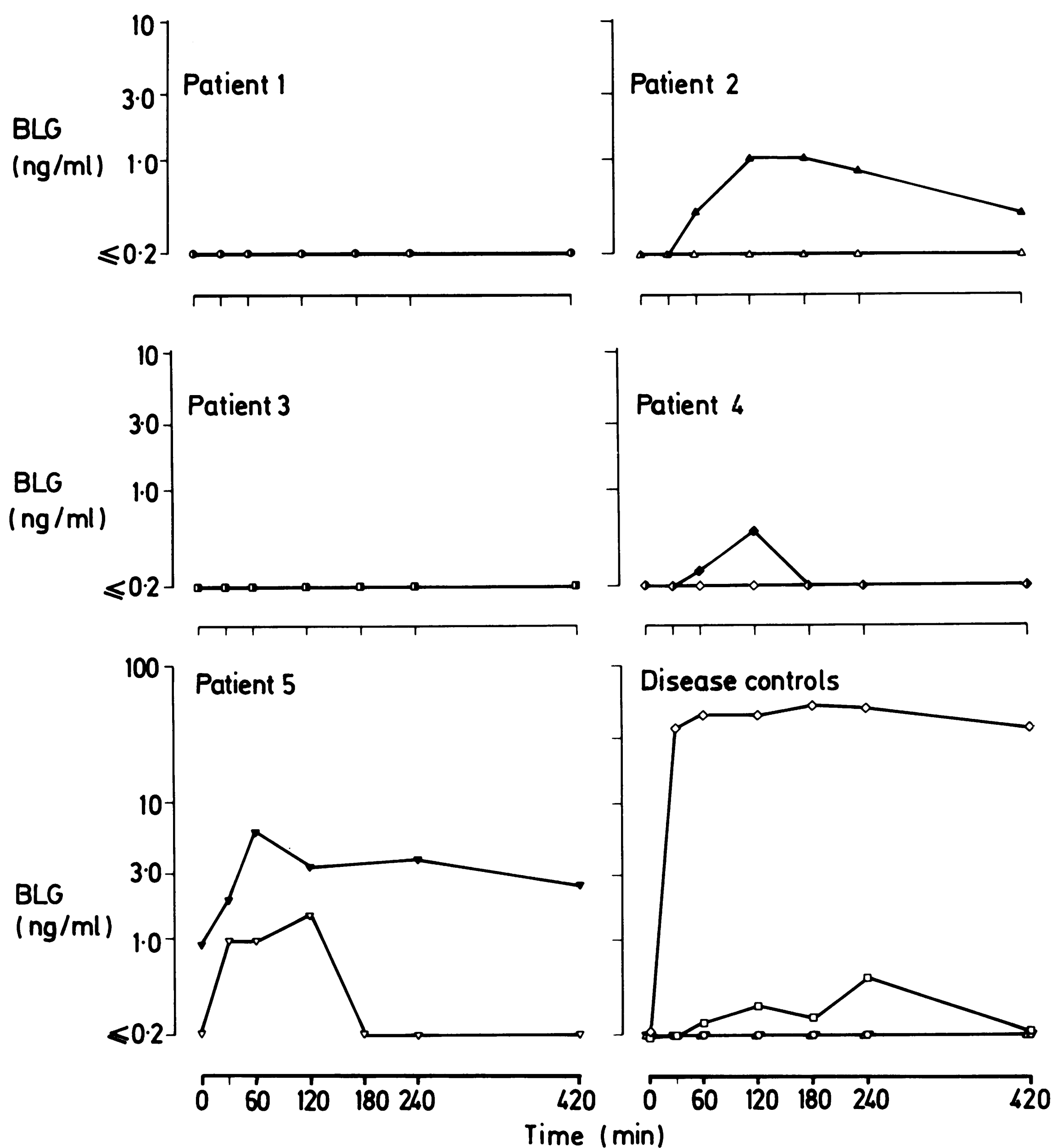

Fig 2 *Beta-lactoglobulin (BLG) in sera from five children with coeliac disease, on a gluten free diet (open symbols) and after gluten challenge (closed symbols) and in five disease controls. Coeliac patients nos 2 and 3 did not have detectable BLG in serum. Note logscale of the ordinate.*

BLG in the t_0 sample obtained after gluten challenge. Peak levels of BLG were found at 60–240 minutes after the meal.

Beta-lactoglobulin was detectable in two disease controls (Fig. 2, disease controls) one of which already showed a maximal level of 50 ng/ml serum 30 minutes after the meal.

ANALYSIS OF THE SIZE DISTRIBUTION OF OA AND BLG

Low levels of OA and BLG were observed after fractionation of t_0 sera from both coeliac patients and controls (data not shown). All individuals had demonstrable amounts of OA and BLG in serum after the test meal, detectable by ELISA in combina-

tion with HPLC. The amounts of OA detected in four of five of the coeliac children were clearly higher at provocation with gluten than after one year on a diet (Fig. 3). The fifth coeliac child (no 1), however, had larger amounts of OA after the diet period.

In four of five coeliac children (nos 2, 3, 4, and 5) the anti-OA reactive material was located mainly in the high MW fractions. The high-MW OA in one patient (no 3) eluted at the V_0 (about 660 kD), whereas the aggregated OA in the rest of the coeliac children was of a smaller size. In contrast, in one patient (no 1) most of the OA eluted at the V_e of native OA (Fig. 3).

Ovalbumin was observed in high MW fractions in five of five control children (Fig. 3, disease controls), and in addition eluted at the V_e of native OA in 2/5 controls. The MW of the OA aggregates was below V_0, except in one case (control no 5).

In four of the five coeliac children BLG was detected by HPLC-ELISA, in larger amounts after gluten challenge than after gluten free diet (Fig. 4). Beta-lactoglobulin was observed in the high MW-fractions in all the coeliacs (nos 1–5), predominantly between V_0 and the elution volume of IgG. Two patients (nos 1 and 2) with no detectable BLG in unfractionated serum had demonstrable high MW BLG after HPLC fractionation. Patient no 1, in which the gut mucosa had not completely normalised after a year of gluten free diet, had low comparable amounts of BLG after the diet period and at gluten challenge.

In the disease controls group (Fig. 4, disease controls) BLG was demonstrated in the high MW fractions only, except for one child (disease control no 1), whose serum also contained BLG fragments.

ANTIBODY DETERMINATIONS

Antibodies were in all cases determined on fasting sera. Levels of IgG anti-OA antibodies were higher in the coeliac children than in the disease controls (Table 3), and so were IgA anti-OA antibodies except in the IgA-deficient individual, whereas the anti-OA antibody titres as determined by the Farr type assay overlapped. The IgA and IgG anti-OA antibodies increased upon gluten challenge in the coeliac children but there was no clear relationship between the degree of OA uptake in serum and serum antibody levels. The coeliac child with IgA-deficiency (no 2), however, had a high concentration of OA in serum, and patient no 4 with no OA in serum had the highest IgG and IgA anti-OA levels in serum.

The IgA anti-BLG antibody titres tended to be higher in coeliac children than in disease controls, whereas the corresponding IgG antibodies (ELISA) and the concentration of anti-BLG antibodies as determined by a Farr type assay were similar in coeliac children and in controls (Table 4). The IgA antibodies to BLG increased in the coeliac children, whereas the IgG antibodies and the antibodies determined by the Farr assay showed a more variable response to gluten challenge. Two of the three coeliac children with detectable BLG in ELISA and one control with a high uptake of BLG (50 ng/ml serum) had low IgG and IgA antibody levels.

Discussion

This investigation showed the presence of OA in serum of nine of 10 children (four coeliacs and five controls) and BLG in five of the children (three coeliacs and two controls) after a test meal. Similar observations have been made previously in a study[17] using radioimmunological techniques. When ELISA-analysis for antigen was combined with fractionation of serum by HPLC, OA and BLG was detected in all serum samples shown to contain these antigens in the direct ELISA. In addition, when no antigen was measured by the direct assay, the combined use of HPLC and ELISA permitted the detection of OA and BLG in the samples obtained three hours after the test meal.

The appearance of the antigens in serum was investigated in each coeliac child after a gluten free diet period and at gluten challenge. The antigen levels showed an increase after gluten challenge in four of five coeliac children. In the one patient with the reverse pattern of OA uptake and no differences in BLG concentrations before and after gluten challenge (no 1, Figs. 1 and 4) the histology of the jejunal mucosa was not completely normalised after the diet period. Thus, the uptake of OA and BLG tended to increase (in eight of 10 samples), when the mucosa exhibited villous atrophy.

The permeability of the gut to macromolecules may be increased in coeliac disease because of a greater leakiness or decreased due to a diminished absorptive area. The mucosal damage may affect both intercellular passage and intracellular transport, the latter probably being the most important under physiological circumstances.[23] The partly conflicting results obtained when estimating gut permeability by use of different non-protein probes[13–15] may reflect the uptake of the probes through different routes. Interestingly, the probe PEG 400 with the largest MW showed a decreased absorption in coeliac disease, perhaps because of the lipid soluble properties of PEG.[16] These results are at variance with the present findings based on measurements of protein antigens.

Analysis of consecutive serum samples revealed the appearance of dietary antigens in serum as early as 30 minutes after the test meal. Paganelli, *et al*[1,7]

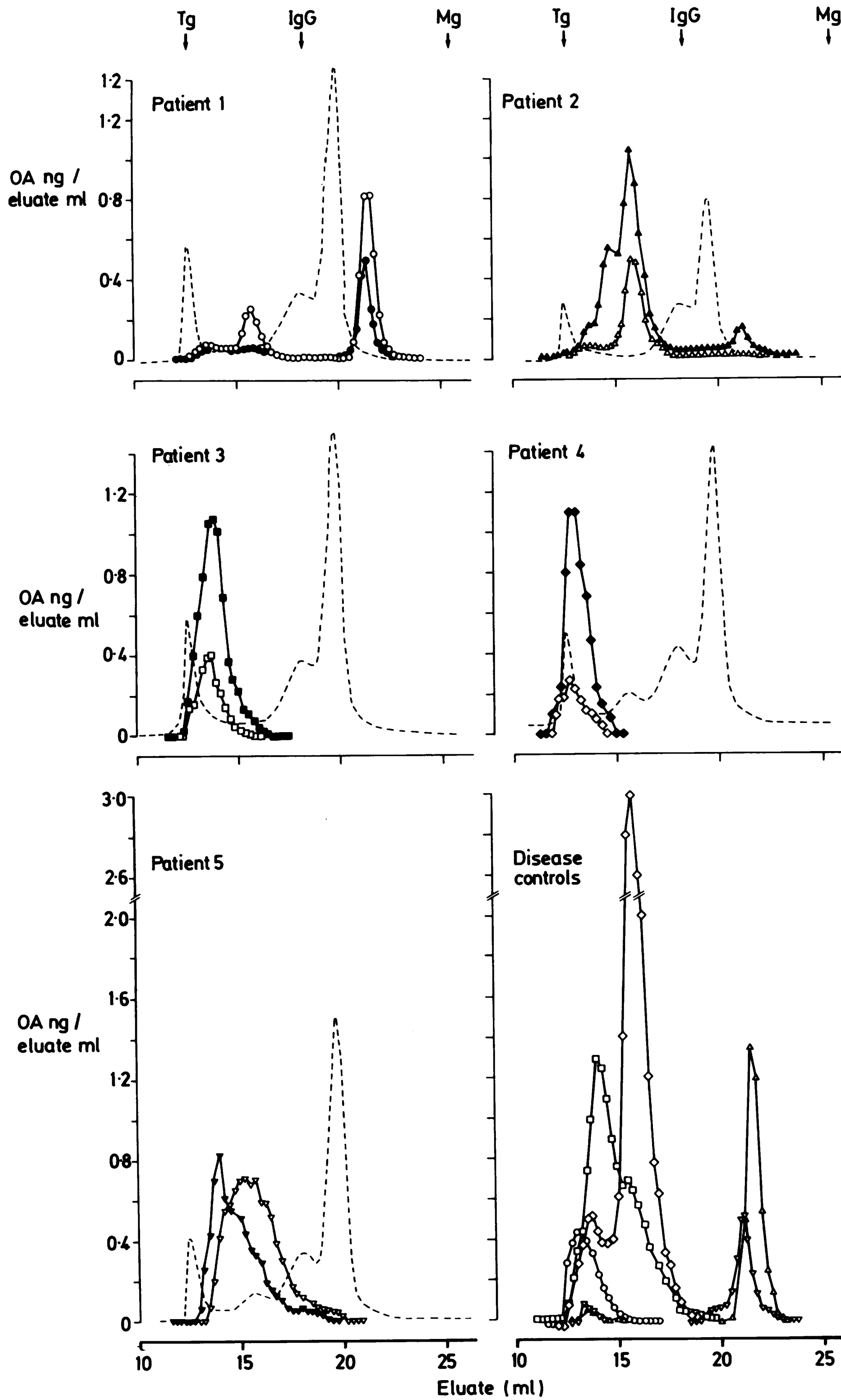
Tg
IgG
Mg
Tg
IgG
Mg
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Disease controls
OA ng / eluate ml
OA ng / eluate ml
OA ng / eluate ml
1·2
1·2
0·8
0·4
0
3·0
2·6
2·0
1·6
0·8
10
15
20
25
Eluate (ml)

Table 3 *Maximal OA concentrations and anti-OA antibody levels in serum of coeliac children and disease controls. Values from coeliac patients were those obtained after challenge with gluten. Symbol used for each child in the figures is denoted in parentheses*

Coeliac children	*Max OA levels (ng/ml)*	*Serum anti-OA (titre)*	*IgG anti-OA† (mU/ml)*	*IgA anti-OA‡ (titre)*
No 1 (●)	15·0	31	640	1130
2§ (▲)	178	200	1000	<50
3 (■)	8·0	270	680	2350
4 (▼)	—	48	1475	12000
5 (◆)	9·0	33	325	530
Disease controls				
No 1 (◇)	91	130	132	<50
2 (□)	42	110	195	400
3 (▽)	5·2	150	11	200
4 (△)	10·5	26	12	<50
5 (○)	3·8	75	175	400

*Measured by a modified Farr assay; †Measured by ELISA and related to a high-titered human serum (1000 mU/ml); ‡Measured by ELISA, titre expressed as the highest dilution giving a signal above background; §IgA-deficient.

reported a diphasic uptake of BLG in the sera of adults and children, hypothesising uptake both through the lymphatic and the local venous system. Such a diphasic uptake was not observed in the present study. Furthermore, seven of nine individuals with detectable OA in serum and three of five with BLG in serum showed no clear antigen peak over a seven hour period after the meal. This suggests a slow metabolism of the antigen resembling our results in healthy adults, some of whom had detectable OA aggregates in the circulation as late as 36–48 hours after a test meal.[24]

We did not in this limited study observe any clear relationship between maximum levels of BLG and OA in serum and antibody titres as measured by Farr assay or ELISA's for IgG or IgA antibodies (Tables 3 and 4). A high uptake of the antigens, however, was observed in the individual suffering from coeliac disease combined with IgA-deficiency. Our previous study[24] of healthy adults indicated a positive relationship between the presence of high-MW OA and IgG anti-OA antibody levels. The majority of the children studied here had only high MW and no free OA or BLG in their sera, probably because of the higher antibody levels observed in childhood in general,[25] and in coeliac children in particular. It should be borne in mind, however, that our disease controls were not healthy children.

Fig. 3 *HPLC fractionation (TSK G-3000 SW) in combination with ELISA for ovalbumin (OA). Results from the five children with coeliac disease, on a gluten free diet (open symbols) and after gluten challenge (closed symbols) and in five disease controls. Patient numbers (nos 1–5) are denoted in the figure. Serum samples analysed were those collected at maximal levels of OA as detected by the direct ELISA, or at 180 mins after the test meal. Broken line denotes fractionation pattern (E_{280}) of the serum obtained at gluten challenge. The elution volumes of thyroglobulin (Tg), IgG and myoglobin (Mg) are indicated for patients nos 1 and 2. Symbols as in Figs 1 and 2.*

Circulating immune complexes have been reported in patients with coeliac disease.[26,27] In the present study antibody reactive material was present in the high MW fractions both in coeliac patients and in the controls. In healthy adults the anti-OA reactive material in the high MW fractions from the HPLC column was shown to contain acid dissociable antigen-antibody complexes.[3] When OA or BLG antigen was detectable only after HPLC fractionation of sera the antigenic material was demonstrable solely in the high MW fractions. The chromatography possibly caused rearrangements of the antigen antibody complexes, exposing epitopes due to changes in the buffer matrix or to dilution effects thus allowing for the detection of these complexes. A major part of the immune complexes had an apparent size below 660 kD (V_0), which indicates a restricted antigen antibody composition. The amounts of high MW antigen detected were far below the detection limit of antigen non-specific assays for immune complex detection, but dietary antigens may be one of the constituents of immune complexes found in earlier studies.[26,27] Antibody bound enteric antigen has been reported in the serum from adult coeliac patients, suggesting that an autoimmune reaction against the villous mucosa may occur in coeliac disease.[28]

Free OA and BLG eluted with M_r corresponding to the native proteins with one exception for BLG,

Table 4 *Maximal BLG concentrations and BLG antibody concentrations in serum. Values from coeliac patients were those obtained after gluten challenge. Symbol used for each child in the figures is denoted in parentheses*

Coeliac children	*Max BLG levels (ng/ml)*	*Serum anti-BLG* (titre)*	*Ig anti-BLG† (mU/ml)*	*IgA anti-BLG‡ (titre)*
No 1 (●)	—	42	400	300
2§ (▲)	1·0	50	145	<50
3 (■)	—	210	1900	680
4 (▼)	0·6	27	1000	430
5 (◆)	6·0	23	84	440
Disease controls				
No 1 (◇)	50	40	13	<50
2 (□)	0·5	50	155	210
3 (▽)	—	38	32	<50
4 (△)	—	34	60	200
5 (○)	—	96	550	450

*Measured by a modified Farr assay; †Measured by ELISA; ‡Measured by ELISA; §IgA-deficient.

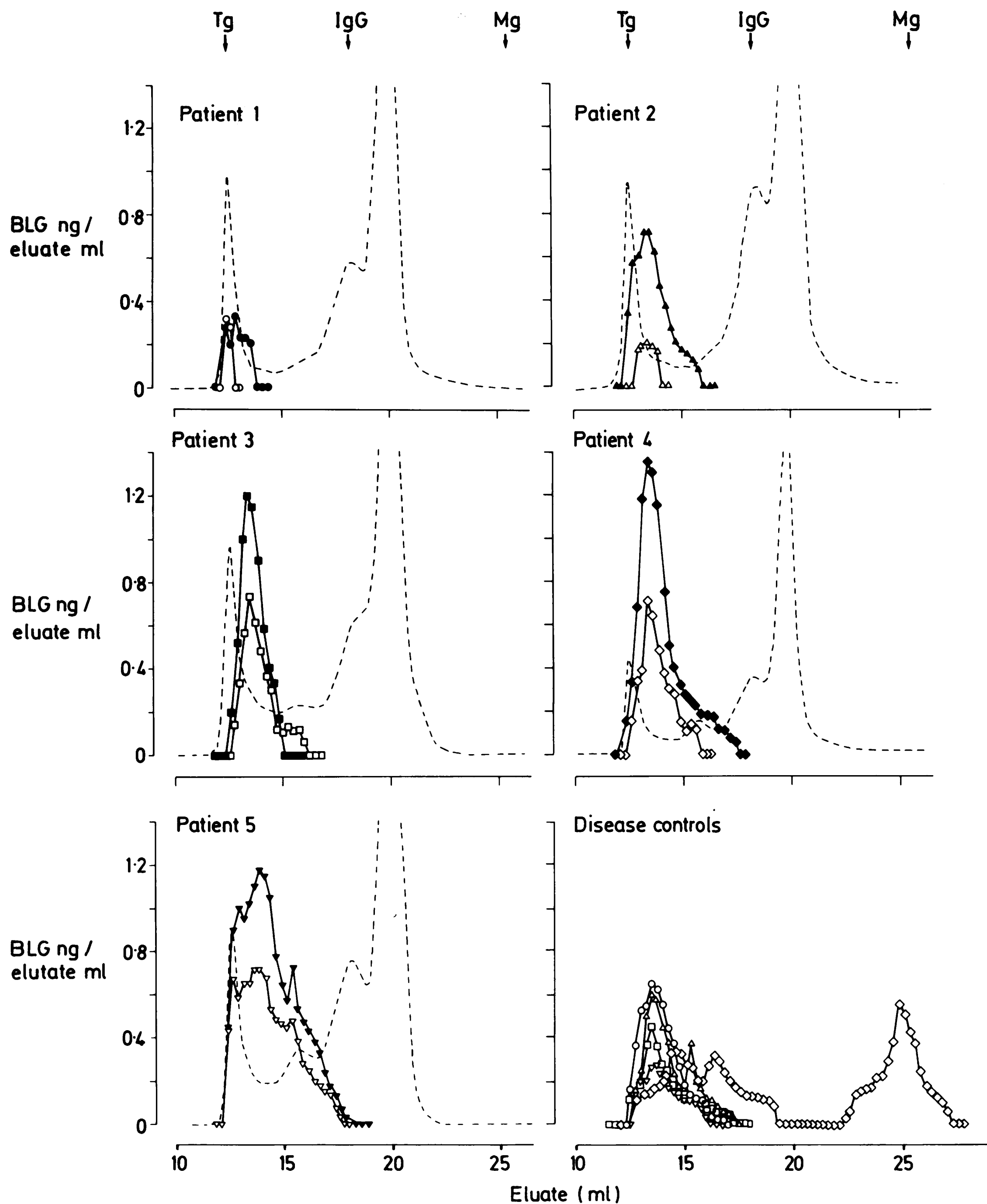

Fig. 4 *HPLC-fractionation in combination with ELISA for beta-lactoglobulin (BLG). Results from the five children with coeliac disease, on a gluten free diet (open symbols) and after gluten challenge (closed symbols) and in five disease controls. Analysed serum samples were those taken at maximal BLG levels as measured by the direct ELISA, or at 180 mins after the meal. Broken line denotes fractionation pattern (E_{280}). Elution volumes of purified protein markers as in Fig. 4. Symbols correspond to those in Figs 1, 2, and 4, and patient numbers are indicated in the figure.*

which was detected mainly in the form of fragments (MW about 17 kD) corresponding to one monomer of the dimeric BLG molecule (Fig. 4, disease controls). This observation for OA as in accordance with our findings in healthy adults.[3] Either the rabbit antibodies raised against the intact proteins do not recognize the epitopes on putative fragments or the fragments are rapidly degraded. Because BLG-fragments were detectable in one individual, the latter explanation seems more likely.

The human gastrointestinal tract allows a certain degree of physiological absorption of undegraded macromolecular dietary antigen, free or antibody bound. This antigen uptake is influenced by the permeability of the gut and the local and systemic immune responses. The present study indicates that challenge with gluten and subsequent development of villous atrophy in coeliac children leads to increased uptake of macromolecular dietary antigens.

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