Cellular hypersensitivity to gluten derived peptides in coeliac disease

R GUAN, P M RAWCLIFFE, J D PRIDDLE, AND D P JEWELL

From the Gastroenterology Unit, Radcliffe Infirmary, Oxford

SUMMARY Wheat gluten derived antigens have been tested for their ability to inhibit the migration of leucocytes from healthy subjects and patients with coeliac disease. Three preparations of a water soluble fraction (Frazer’s fraction III, FIII) of partial peptic trypsic digests of wheat gluten had different effects in a direct (one stage) assay. Subfractions B and B2 caused migration inhibition of leucocytes from patients with treated coeliac disease but not of leucocytes from healthy volunteers or patients with Crohn’s disease or ulcerative colitis. This migration inhibition seems to be specific for gluten fractions because maize zein fraction B, β-lactoglobulin and ovalbumin did not cause it. The sensitivity of coeliac leucocytes to fraction B is not related to factors present in coeliac serum as the migration of leucocytes from healthy individuals preincubated with coeliac sera was not inhibited. Puromycin diminished inhibition by fraction B, which was active at 1·2 µg/ml in an indirect (two stage) migration inhibition assay; this is consistent with a process involving elaboration of lymphokine(s). More highly purified fractions of B2, P1–P4 were prepared by reverse phase high performance liquid chromatography (HPLC) and showed differing potency in direct and indirect assays, with P4 being the most active fraction. Inhibition of migration by gluten derived peptides appears to result from the release of lymphokine by leucocytes specifically from coeliac patients.

Since Dicke1 first established the toxicity of wheat gluten in patients with coeliac disease there have been numerous attempts to identify its toxic component. Frazer et al2 showed that a water soluble fraction (fraction III, FIII) of a partial peptic trypsic digest of gluten retained its damaging properties. Dissanayake et al3 separated FIII into three fractions (A, B, and C) by ultrafiltration. By feeding studies, in volunteer patients with treated coeliac disease, they showed that fraction A (lowest molecular weight) was not harmful, but that fractions B and C produced histological damage. Anand et al4 separated fraction B into further subfractions by gel filtration on Sephadex G50. Of the three subfractions separated, those with the higher molecular weights (B2 and B3) were found to be toxic by feeding. Working with intact gliadins, de Rooij et al5 have shown the toxicity of alpha gliadins by instillation and Ciclitira et al6 have demonstrated the toxicity of alpha, beta, gamma, and omega gliadins.

The ultimate test of toxicity remains the demonstration of histological damage in vivo, but as fractions are progressively purified it becomes increasingly difficult to prepare sufficient quantities for feeding experiments in coeliac patients and in vitro experiments become necessary. While the mechanism by which gluten produces mucosal damage in patients with coeliac disease is unknown, there is now a considerable body of evidence implicating immunological mechanisms.

Several groups have studied cellular immune reactions to gluten fractions in vitro, principally by the leucocyte migration inhibition assay which is usually held to be a measure of cell mediated immunity.7-30 While much migration inhibition testing has been done by the capillary tube method, it is more difficult to perform and less sensitive than the
agarose gel technique described by Clausen. The following experiments were carried out by the latter method to determine the cellular responses to the gluten fractions FII, B and B2, and to confirm the specificity of these responses for coeliac disease.

More recently, fraction B2 has been further separated using reverse-phase HPLC. The peaks fell into four main groups, designated P1 to P4, each containing several peptides. Results using these peptide fractions in both the direct and the indirect leucocyte migration inhibition assay are described.

Methods

Patients

Twenty two patients with coeliac disease were studied. All had villous atrophy consistent with coeliac disease at the time of presentation, and all had a good clinical and histological response to a gluten free diet and remained well on the diet at the time of testing.

Two groups of similar age and sex distribution were chosen as controls; 13 hospital staff and 10 patients with quiescent inflammatory bowel disease (one with chronic colitis of undetermined type; four with ulcerative colitis; and five with Crohn’s disease – of whom two had colitis, one had terminal ileal disease, and two had both ileal and colonic disease). No patient was being treated with corticosteroids or immunosuppressives.

Gluten Fractions

Fraction II from a peptic tryptic digest of gluten was prepared by the method of Frazer et al in Leeds (from commercial gluten derived from a mixture of wheat varieties, British Drug Houses) and in Oxford (from Flanders and Scout 66 wheats).

Trypsin inactivated FIII (Flanders) was prepared by heating FIII to 90°C for 30 minutes. The absence of tryptic activity was confirmed by the method of Rick.

Fraction B and fraction B2, were prepared from Scout 66 wheat according to the methods of Dissanayake et al and Anand respectively. This preparation of fraction B has been found to produce mucosal damage in feeding experiments and the B2 used in the present study was made from it. The peptide groups P1 to P4 were derived by reverse phase HPLC from a new sample of B2, as described by Rawcliffe et al.

Two preparations of gliadins were made from Flanders wheat. The first was prepared by Sephacryl S200 gel filtration to remove omega gliadins and glutenins and to yield alpha/beta/gamma gliadins. This preparation was subjected to CM52 ion exchange chromatography in urea containing buffers to give a second preparation designated ‘late eluting’ gliadins (predominantly alpha gliadins by starch gel electrophoresis, details of this unpublished work are available on request). Purified protein derivative of human tuberculin (Central Veterinary Laboratory, Surrey, England) was used as a positive control antigen.

Maize zein fraction B (prepared in a similar manner to wheat gluten fraction B), bovine β-lactoglobulin (Sigma), and chicken ovalbumin (Pharmacia) were also used as test antigens.

Direct Leucocyte Migration Inhibition Assay

This was carried out by the method of Clausen. 30 ml heparinised blood was mixed with 8 ml 6% w/v Dextran 150 (MW 150000) in 0-9% NaCl (Fisons, Loughborough, England) and left to sediment at 37°C for 45 minutes. The buffy coat was removed, washed three times with Hanks’ Balanced Salt Solution (HBSS; Gibco, Paisley, Scotland) and resuspended in RPMI 1640 culture medium with 25 mM Hepes and L-glutamine (Gibco) containing 10% heat inactivated fetal calf serum (Gibco). The leucocyte yield was usually about 100×10^6 (range 50–130×10^6) and consisted of approximately 80% granulocytes and 20% lymphocytes. The erythrocyte:leucocyte ratio was 1:1.

Agarose (Indubiose A45; Uniscience Ltd, London SW18 2LS, England) was added to sterile water, boiled to dissolve it and cooled to 47°C. At that temperature horse serum (Wellcome, Beckenham, England) and 10× concentrated TC199 culture medium (Wellcome) were added to a final concentration of 10% each. Final concentration of agarose was 1%. 7-5% NaHCO₃ solution (Flow Laboratories, Rickmansworth, England) was added so that the pH of the medium after incubation in 5% CO₂ in air was between 7-2 and 7-4. Penicillin (66 IU/ml; Glaxo, Greenford, England) and streptomycin (66 μg/ml; Evans Medical, Greenford, England) were also included. 10 ml agarose – serum – TC199 – NaHCO₃ solution were then poured into disposable 9 cm diameter plastic Petri dishes and wells of 2 mm diameter were punched out in the gel after it had solidified.

The leucocytes were preincubated with antigens as follows: aliquots of the cell suspension were placed in 1-5 ml plastic centrifuge tubes, antigen was added and the mixture incubated for 30 minutes at 37°C. Antigens were tested at final concentrations from 10 μg/ml to 1 mg/ml. Phosphate buffered saline was added to the control suspension instead of antigen. Cell viability after incubation was assessed by Trypan blue dye exclusion and 5 μl of each suspension (final cell concentration 300×10^6/ml) were placed in the agarose wells. Most samples were assayed in quad-
replicate (triplicate in a few cases) and, if more than one plate was used in testing an individual, control wells were included in each plate. The Petri dishes were incubated for 18 hours at 37°C in a humidified atmosphere of 5% CO₂ in air. The cells were fixed with 95% alcohol for six hours or more. The agarose was then gently lifted from the dishes and the areas of migration projected onto a Kontron MOP-AMO3 electronic area measurement tablet. This was programmed to measure the area of the image outlined by a measuring pen.

A migration index was calculated:

\[
\text{Migration index (MI)} = \frac{\text{Mean migration area of four wells with antigen}}{\text{Mean migration area of four control wells}}
\]

Results were analysed statistically by the unpaired Student's t test.

Puromycin (Sigma) at a final concentration of 15 \( \mu \text{g/ml} \), together with fraction B at 100 \( \mu \text{g/ml} \), was incubated with leucocytes from four coeliac patients.

**Indirect Leucocyte Migration Inhibition Assay**

Heparinised peripheral venous blood, diluted with an equal volume of HBSS, was layered onto a Ficoll-Paque (Pharmacia) gradient and centrifuged at 1200 rpm for 35 minutes. The mononuclear cells at the interface were removed, washed twice with HBSS and resuspended at 1.5×10⁶ cells/ml in RPMI 1640 containing 10% fetal calf serum. 1 ml aliquots were dispensed into upright plastic culture tubes with or without 1.2 \( \mu \text{g/ml} \) of fraction B.

Cultures were incubated for 72 hours, the cell suspensions centrifuged and the supernatant diluted 1:1 with fresh RPMI 1640. The unstimulated culture supernatants were reconstituted with appropriate concentrations of antigens. Leucocyte suspensions, prepared by dextran sedimentation from the blood of healthy donors, were used as indicators for measurement of migration inhibitory activity in these supernatants. For the indirect test, migration index (MI) was calculated as follows:

\[
\text{Migration index (MI)} = \frac{\text{Mean migration area of four wells with supernatant from cells incubated with antigen}}{\text{Mean migration area of four wells with supernatant from control cells reconstituted with antigen}}
\]

**Serum Preincubation**

Ten microlitre aliquots of buffy coat cells (300×10⁶/ml) from a healthy individual were incubated at 37°C for one hour with 20 μl serum from eight coeliac patients and from five normal individuals. The cells were then washed twice and used in the direct migration inhibition assay with fraction B as antigen (100 μg/ml).

![Graph](image)

**Fig. 1** Effect of FIII (Leeds) on leucocyte migration in normal individuals, patients with coeliac disease and patients with ulcerative colitis or Crohn's disease (disease controls), at doses of 10 μg/ml and 1 mg/ml. ○= healthy control. ●= coeliac disease. △= disease control.
Gluten peptides in coeliac disease

Results

The areas of migration were well defined. There was less than 10% variation between the areas of migration of quadruplicate wells. Preliminary experiments showed that the presence of erythrocytes or slight variation of pH from 7·2 to 7·4 did not interfere with the test. There was no difference in Trypan blue dye exclusion by cells incubated with or without antigens (except in the cases with high doses noted below), indicating that cell death was not the cause of any inhibition of migration.

Using purified protein derivative as antigen, leucocytes from patients with coeliac disease behaved like those from healthy individuals, migration being depressed by 100 μg/ml purified protein derivative (healthy controls (n=8), mean migration index = 0·76; coeliac patients (n=19), mean migration index = 0·78).

Frazer's fraction III prepared in Oxford from Flanders wheat as well as from Scout 66 wheat had no effect on the migration of leucocytes from either healthy individuals or patients with coeliac disease at concentrations as high as 1 mg/ml (Flanders fraction III: healthy controls (n=7), mean migration index = 1·12; coeliacs (n=14), mean migration index = 1·08; no significant difference, p>0·1; Scout 66 fraction III: healthy controls (n=7), mean migration index = 1·10; coeliacs (n=7), mean migration index = 1·08; no significant difference, p>0·5) (Fig. 1). Flanders fraction III, however, showed a lower migration index after inactivation of trypsin with both normal (p<0·02) and coeliac (p<0·05) leucocytes.

Fraction III from Leeds inhibited leucocyte migration in patients with coeliac disease at 1 mg/ml (compared with healthy controls, p<0·001). Significant inhibition was seen even at 10 μg/ml (compared with healthy controls, p<0·001). This antigen also inhibited the migration of leucocytes from patients in the disease control group at 1 mg/ml (Fig. 1).

Alpha/beta/gamma gliadins (100 μg/ml and 1 mg/ml) failed to inhibit the migration of leucocytes from either coeliac patients or normal individuals (100 μg/ml: healthy controls (n=7), mean migration index = 0·96; coeliacs (n=6), mean migration index = 0·98. 1 mg/ml: healthy controls (n=7), mean migration index = 0·99; coeliacs (n=6), mean migration index = 0·92). 'Late-eluting' gliadins, however, tended to inhibit migration of leucocytes from coeliac patients at 100 μg/ml, although this did not reach statistical significance at the 5% level (10 μg/ml: healthy controls (n=11), mean migration index = 1·07; coeliacs (n=9), mean migration index = 1·01; p>0·1. 100 μg/ml: healthy controls (n=11), mean migration index = 1·00; coeliacs (n=11), mean migration index = 0·89; 0·1>p>0·05).

Fractions B and B2 of Scout 66 wheat had no effect on the migration of leucocytes from healthy subjects (Fig. 2). For fraction B the largest difference between patients and healthy controls was at 10 μg/ml (p<0·01). For subfraction B2 the difference was greatest at 100 μg/ml (p<0·001) (Fig. 3). The migra-
Faction index for normal controls fell below 1·0 at the highest dose of 1 mg/ml when a reduction in cell viability was seen after the preincubation period.

The migration of leucocytes from patients with coeliac disease was not significantly inhibited by zein fraction B (100 μg/ml: healthy controls (n=8), mean migration index=1·09; coeliacs (n=12), mean migration index=1·05), nor by β-lactoglobulin (100 μg/ml: healthy controls (n=8), mean migration index =1·01; coeliacs (n=12), mean migration index=1·08), nor by ovalbumin (100 μg/ml: healthy controls (n=6), mean migration index=0·99; coeliacs (n=10), mean migration index=1·06).

Puromycin at 15 μg/ml diminished migration inhibition by fraction B at 100 μg/ml in three of four coeliac patients.

The results of an indirect assay with fraction B are shown in Figure 4. The migration of normal leucocytes is inhibited by culture supernatants of peripheral blood mononuclear cells from patients with coeliac disease incubated with fraction B at 1·2 μg/ml. In contrast, the results shown for the direct assay are with 80 times as much antigen.

Preincubation of normal leucocytes with sera from eight patients with coeliac disease did not sensitize these cells to the antigen, fraction B at 100 μg/ml (mean migration index of normal leucocytes exposed to coeliac sera=0·94; mean migration index of normal leucocytes exposed to normal sera=1·04; not significantly different, p>0·1).

A comparison of the results of the direct migration inhibition assay using gluten fraction B2 of Anand et al9,10 and the new fraction B2 shows that these two fractions both inhibit the migration of leucocytes from coeliac patients to the same extent. Leucocytes from healthy subjects show very little inhibition with either fraction. In the disease controls there was no

Fig. 3  Migration indices of leucocytes from healthy individuals and patients with coeliac disease exposed to different concentrations of gluten fraction B2. Error bars show SEM.

Fig. 4  Effect of fraction B in the indirect leucocyte migration inhibition assay. Filled circles (●) show the migration indices in the direct assay for leucocytes from six coeliac patients incubated with fraction B at 100 μg/ml. Peripheral blood mononuclear cells from the four patients with the lowest values in the direct assay were incubated with fraction B at 1·2 μg/ml and the effect of the culture supernatants on the migration of normal leucocytes tested in the indirect assay is shown as open squares (□). Lines connect the results in the two different assays for the same patient.
Gluten peptides in coeliac disease

Fig. 5  Migration indices of leucocytes from normal individuals, patients with coeliac disease, and patients with inflammatory bowel disease (disease controls) after exposure to peptide groups from RP-HPLC, at 100 μg/ml. P values compare coeliac disease patients with healthy controls.

Figures 5 and 6 show the migration indices of leucocytes from normal individuals, patients with coeliac disease and disease controls exposed to 10 μg/ml and 100 μg/ml of each of the peptide groups P1 to P4. There was significant migration inhibition of leucocytes from coeliac patients exposed to all four peptide groups. The migration indices in coeliac patients were lowest with fraction P4 (compared with healthy controls, 100 μg/ml: p<0.001, 10 μg/ml: p<0.01).

When leucocytes from healthy individuals were exposed in the indirect assay to the culture supernatants from mononuclear cells of five coeliac patients that had been incubated with antigens, their migration was inhibited (Fig. 7). The antigens used to stimulate the coeliac cells were P1 and P3 at 1.2 μg/ml.

Discussion

The inhibition of migration of leucocytes by purified protein derivative in both healthy individuals and
patients with coeliac disease suggests that the lymphocytes from the patients retain their ability to produce migration inhibition factor(s) when stimulated with recall antigens.

FIII has been shown to be toxic to the coeliac intestinal epithelium by feeding experiments (reference 2 and, for Scout 66, P M Rawcliffe, unpublished work). FIII has also been reported to cause inhibition of migration of leucocytes in patients with coeliac disease.7 O’Farrelly et al.,15 however, failed to confirm this response. Four preparations of FIII were tested in the present study and a positive response found to the preparation from Leeds but not to those prepared in Oxford. Gel filtration on Sephadex G-50 in Oxford has shown the presence of large molecular weight material in the preparation from Leeds which was not found in the local FIII (P M Rawcliffe and J D Pridde, unpublished observations). This may represent large fragments of gluten and could explain a recent report from Leeds that migration inhibition of coeliac leucocytes by FIII (Leeds) might not be caused by lymphokine activity.16 It is conceivable that the large molecular weight material in the Leeds preparation could bind to cytophilic antibodies present on the leucocytes of patients with coeliac disease. This mechanism could explain the low migration indices with this antigen in patients in our disease control group. Although direct cytotoxicity cannot be ruled out completely by Trypan blue exclusion, it is probably not the reason for the observed low migration index in these patients as healthy individuals showed no migration inhibition with the same dose of the antigen (Leeds FIII, 1 mg/ml).

Gliadins have been held to induce cell mediated immunity in coeliac patients.7 Alpha/beta/gamma gliadins prepared in Oxford failed to inhibit the migration of leucocytes from patients with coeliac disease. O’Farrelly and coworkers19 obtained a similar result with mixed gliadins, using the capillary tube method. They found, however, migration inhibition with coeliac leucocytes exposed to purified gliadins...
Gluten peptides in coeliac disease

alpha-, beta-, and gamma-gliadins separately. Migration inhibition with 100 μg/ml of ‘late-eluting’ gliadins prepared in Oxford did not reach statistical significance and experiments with larger doses were not done.

We believe it is important that inhibition of the migration of leucocytes from coeliac patients has been demonstrated for fraction B which causes histological damage to the coeliac small intestinal mucosa in vivo. The sensitivity of coeliac leucocytes to fraction B is unrelated to factors present in coeliac serum. Gluten fraction B is effective in both direct and indirect migration inhibition tests at very low doses, and its action is abolished by puromycin. This suggests that the cellular hypersensitivity of patients with treated coeliac disease to gluten peptides is mediated by soluble factors (lymphokines). β-lactoglobulin, ovalbumin and maize zein fraction B did not cause migration inhibition of leucocytes from patients with coeliac disease, so the observed responses to gluten may be of considerable significance in the pathogenesis of the disease, rather than merely an immune response consequent on loss of mucosal integrity.

Gluten is a complex material which contains many proteins with closely similar physical characteristics and overall chemical composition. The available evidence suggests that they show a considerable degree of structural homology, both between and within individual gliadin molecules. The result of an incomplete pepsin tryptic digest of gluten under the conditions of Frazer et al is yet more complex.

Fractions B and B2 inhibit migration of coeliac leucocytes while the preparation of Scout 66 FIII from which they derive does not. Ashkenazi and coworkers18 found that migration inhibition was more marked when leucocytes from coeliac patients were incubated singly with either their B2 or their B3 subfraction than when they were incubated with a mixture of the two or with their FIII. They speculated that fractionation of FIII into smaller peptides uncovers immunogenic sites which are again masked by remixing these fractions.

We have shown no effect of fractions B and B2 in healthy controls. Other workers19 have noted cell mediated immunity to our fraction B2 in non-coeliac patients with active small bowel diseases.

Fraction B2 has now been separated into four peak groups,20 each still containing many peptides, by reverse phase HPLC. All four peptide groups caused migration inhibition of leucocytes from patients with coeliac disease in the direct assay, at low concentrations. P4 was the most active. This effect was only seen with coeliac patients. The ability of culture supernatants to inhibit the migration of leucocytes from normal individuals suggests that lymphokine is produced by the stimulation of mononuclear cells from patients with coeliac disease by gluten derived peptides.

The indirect test was not performed with peptide groups P2 and P4 because of a lack of materials. Any ‘active sequence’ (or sequences) could be present in many of the peptides resulting from the partial digestion procedure used here. It need not therefore be surprising, at this level of separation, that all four peptide groups, each with a number of peptides, exhibit some degree of activity. They are the subject of further studies.

We are grateful to Dr Peter Howdle, St James’s Hospital, Leeds, for supplying us with their preparation of Frazer’s fraction III. RG was supported by the University of Singapore and the Coeliac Trust; PMR by the Medical Research Council and Welfare Foods (Stockport) Ltd, and JDP by the Wellcome Trust and Welfare Foods Ltd.

References

9 Haeney MR, Asquith P. Inhibition of leucocyte migration by α-gliadin in patients with gastrointestinal disease: its specificity with respect to α-gliadin and coeliac disease. In: McNicholl B, McCarthy CF, Fottrell PF,
Cellular hypersensitivity to gluten derived peptides in coeliac disease.

R Guan, P M Rawcliffe, J D Priddle and D P Jewell

*Gut* 1987 28: 426-434
doi: 10.1136/gut.28.4.426

Updated information and services can be found at:
http://gut.bmj.com/content/28/4/426

**Email alerting service**
*These include:*
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Topic Collections**
Articles on similar topics can be found in the following collections
- Coeliac disease (537)
- Crohn's disease (932)
- Ulcerative colitis (1113)

**Notes**

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/