Coeliac disease: characterisation of monoclonal antibodies raised against a synthetic peptide corresponding to amino acid residues 206-217 of A-gliadin

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Abstract

A dodecapeptide of A-gliadin, which shares amino acid homologies with the E1b protein of adenovirus 12, was used to produce murine monoclonal antibodies. Five monoclonal antibodies were produced and were screened by enzyme linked immunosorbant assay, immunodot assay, and immunoblotting. The antibodies were tested against whole wheat gliadin and its α , β , γ , and ω subfractions, and the prolamins of rye, barley, oats, maize, millet, rice, and sorghum. Four of the five antibodies cross reacted with one or more of the coeliac non-toxic cereals – maize, millet, sorghum, and rice. The monoclonal antibody that did not cross react with these non-toxic cereals, did not recognise Frazer's fraction III, a peptic-tryptic digest of wheat gluten which is known to be toxic. The results suggest that the A-gliadin dodecapeptide shares a region of homology with cereals that do not exacerbate coeliac disease. This study does not support the hypothesis that prior infection with adenovirus 12 is a precipitating factor in coeliac disease.

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Coeliac disease is characterised by malabsorption secondary to damage of the normal villous architecture of the small bowel. The condition is thought to represent an aberrant HLA class II restricted immune response, mediated by antigen specific T cells sensitive to certain cereal peptides. Coeliac disease is exacerbated by ingestion of wheat, rye, barley, and probably oats. The toxic fraction resides in the ethanol soluble storage protein, the prolamin fraction.¹ Related cereals including maize, millet, sorghum, and rice, are not toxic to patients with coeliac disease. Efforts to identify the coeliac toxic amino acid sequence, which presumably acts as a T cell immunogen, have focussed on the wheat prolamin, gliadin, whose four subfractions α , β , γ , and ω , are thought to be toxic to patients with coeliac disease.^{2,3} The complete amino acid sequence of A-gliadin, one of the α gliadins, has been reported.⁴

Kagnoff has reported a region of sequence homology between part of A-gliadin and the 54 kD E1b protein of adenovirus 12.⁵ This homology is found in domain V of A-gliadin,⁴ between amino acid residues 206-217, which shares an identical pentapeptide (residues 211-215) and three other amino acid identities with the E1b protein. It was postulated that prior

infection with adenovirus 12 might predispose genetically susceptible individuals to the later development of coeliac disease. There have been conflicting reports of raised levels of neutralising, circulating antibodies to adenovirus 12 in coeliac patients.⁶⁻⁸ Persistent infection with adenovirus 12 has not been detected in the small bowel of patients with coeliac disease.^{9,10}

We wished to demonstrate whether epitopes contained within the amino acid sequence 206-217 of A-gliadin are specific to coeliac toxic cereals. Monoclonal antibodies have been raised against a synthetic peptide corresponding to this part of A-gliadin and their cross reactivities with prolamins that are toxic and non-toxic to patients with coeliac disease have been investigated.

Methods

PREPARATION OF ANTIGENS

A synthetic dodecapeptide of A-gliadin (residues 206-217) was kindly provided by Dr D Jewell, Radcliffe Infirmary, Oxford,¹¹ and was conjugated to tuberculin purified protein derivative (PPD) to act as a carrier using 0.05% glutaraldehyde. Whole gliadin and its α , β , γ , and ω subfractions were prepared.¹² The purity of the subfractions was checked by aluminium lactate starch gel electrophoresis. Visualisation of the gel, which had been overloaded with protein, showed only minimal cross contamination of the subfractions, with the exception of β gliadin, which may have been contaminated with 5% of α and γ gliadins.³ Prolamins from rye, barley, oats, millet, sorghum, maize, and rice were extracted from their respective flours at a concentration of 500 mg/ml in 50% ethanol. Frazer's fraction III was prepared as described.¹³ The protein contents of the cereal fractions were measured.¹⁴

IMMUNISATION

Balb c mice that had been maintained on a gluten free diet for many generations in order to overcome oral tolerance to gliadin,¹⁵ were primed subcutaneously with 0.1 ml of BCG vaccine. Two, 4, and 6 weeks later, the mice were injected with 30 μ g of the synthetic peptide conjugate in Freund's adjuvant. Murine antisera were screened against unfractionated gliadin by ELISA, as described below. Non-immunised mice on a gluten free diet served as controls. Thirty μ g of peptide conjugate were then injected intravenously.

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TABLE I Titres of the five monoclonal antibodies produced against the synthetic A-gliadin peptide and the control antibody, J13, as determined by ELISA

Clone	Gliadin	α	β	γ	ω	FFIII	Rye	Barley	Oats	Maize	Rice	Millet	Sorghum	BSA
KG9	2048	2048	2048	2048	64	4	16	512	2	128	1	2048	4	—
KG32	2048	512	64	16	1	—	1	8	—	—	—	—	—	—
KG42	256	64	128	128	128	128	64	256	64	64	2	128	16	—
KG53	512	64	64	256	32	—	512	512	64	—	—	—	—	—
KG62	256	16	32	64	16	2	4	2	32	4	4	256	—	—
J13	—	—	—	—	—	—	—	—	—	—	—	—	—	—

FFIII= Frazer's fraction III; BSA= bovine serum albumin.

PRODUCTION OF MONOCLONAL ANTIBODIES
Splenocytes were fused with murine myeloma cells (P3X63/Ag 8.653)¹⁶ using standard techniques.¹⁷ Hybridomas were screened by ELISA against unfractionated gliadin and those giving an optical density (OD) greater than 1.5 were single cell cloned. Clones were screened against unfractionated gliadin and expanded.

ELISA
ELISAs were performed as previously described.¹⁵ Plates were coated with unfractionated gliadin, its α , β , γ , and ω subfractions, rye, barley, oats, maize, millet, or sorghum prolamins, at concentrations of 50 μ g/ml in 70% ethanol. Frazer's fraction III (25 μ g/ml) was dissolved in carbonate-bicarbonate buffer, pH 9.6. Bovine serum albumin (BSA), 50 mg/ml, served as a negative control. An irrelevant monoclonal antibody, J13, to human small intestinal brush border membranes served as a negative control.¹⁸

IMMUNODOT BINDING ASSAYS
These were performed as previously described.¹⁹ Solutions of wheat gliadin and its subfractions, FFIII, rye, barley, and oat prolamins were used at a concentration of 500 μ g/ml of Lowry positive protein. Casein and ovalbumin, 500 μ g/ml, served as negative controls. Extracts of 500 mg/ml of the flours of maize, rice, millet, and sorghum were applied directly to the nitrocellulose sheets irrespective of prolamins content. Monoclonal antibodies were used at a dilution of 1:2. A control antibody was used as above.

SDS-PAGE AND IMMUNOBLOTTING
This was performed as described.²⁰ Seven μ g of antigen were used per track. Blots were probed with monoclonal antibody supernatant at a dilution of 1:2.

Results

IMMUNISATION
Two mice immunised with the synthetic peptide conjugate showed serum titres to whole gliadin of 1:6400 and 1:12 800 compared with a titre of 1:800 for a non-immunised mouse. The mouse with the highest titre was used.

PRODUCTION OF MONOCLONAL ANTIBODIES
The supernatants from 33 of the 108 wells gave positive ELISA readings against unfractionated gliadin; 12 with an OD greater than 1.5 were cloned. Five stable clones originating from five separate hybridomas were obtained.

ELISA
The titres of the five monoclonal antibodies produced against the synthetic A-gliadin peptide and the control antibody J13 are shown in Table I. Figure 1 shows titration curves for three of the

Figure 1: Titration curves determined by ELISA for monoclonal antibodies KG9, KG42, and KG62 against unfractionated gliadin, rye, maize and millet prolamins.

TABLE II Monoclonal antibodies against test and control antigens determined by immunodot assay (+++=strong spot; ++=moderate spot; +=light spot; +/- = trace; -=no reaction)

Clone	Gliadin	α	β	γ	ω	FFIII	Rye	Barley	Oats	Maize	Rice	Millet	Sorghum	Casein	Ovalbumin
KG9	+++	+++	+++	+++	+/-	+/-	+	+	+	++	++	++	++	+/-	-
KG32	++	++	++	+	-	-	-	+/-	+/-	+/-	++	+/-	-	-	+/-
KG42	+++	+++	+++	+++	+++	+/-	+++	++	++	++	++	++	++	+/-	+/-
KG53	++	++	++	++	+/-	-	+	+	+	-	+/-	-	-	-	-
KG62	+++	++	++	++	+	+/-	++	+	+	+	++	++	+	+/-	+/-
J13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

FFIII = Frazer's fraction III.

antibodies. All antibodies cross reacted with gliadin and its α, β, and γ subfractions. Titres against ω gliadin were generally low. The monoclonal antibodies showed no, or very low, titres to Frazer's fraction III, with the exception of KG42. Three of the antibodies – KG9, KG42, and KG62 – showed cross reaction with the non-toxic cereals, particularly millet and maize.

IMMUNODOT ASSAY

The results for the test and control antibodies are presented in Table II. The dot assay obtained for KG42 is shown in Figure 2. All five antibodies cross reacted with gliadin and its α, β, and γ subfractions. Spots obtained with ω gliadin were generally very weak, with the exception of KG42 which gave a strong spot. KG9, KG42, and KG62 cross reacted with non-toxic maize, millet, sorghum, and rice confirming qualitatively the results of ELISA assays. KG53 reacted with gliadin and its subfractions, but did not react with the non-toxic cereals. KG32 showed cross reaction with rice, which was not apparent using ELISA. KG42 gave only a faint trace with Frazer's fraction III, the others did not react.

SDS-PAGE AND WESTERN BLOTTING

Binding was observed between antibody KG9 and rice and millet prolamins; maize prolamins displayed very weak bands and sorghum did not react in this system.

Discussion

Coeliac disease probably represents an aberrant immune response by antigen specific T cells of the small intestine to certain cereal peptides. It has been suggested that a peptide within A-gliadin, which has sequence homology with the E1b protein of adenovirus 12, may be an important epitope in the generation of such an aberrant immune response. We wished to establish whether epitopes within this peptide are specific to those cereal prolamins known to be toxic to patients with coeliac disease.

We have raised five monoclonal antibodies against a synthetic dodecapeptide of A-gliadin. Four of these antibodies showed cross reactivity with cereals that are not toxic in coeliac disease. Some differences were observed between the results obtained using ELISA and those with dot assay, which is considered to be more sensitive than ELISA.²¹ This may be explained, at least in part, by the greater antigen binding capacity of the solid phase, and might explain why some of the antibodies showed reactivity with antigens bound to nitrocellulose which they failed to recognise by ELISA. SDS-PAGE did not show the binding of antibody KG9 to sorghum prolamins which was observed using dot assay. This may be the result of a reduction in antigenicity caused by SDS.

Antibody KG53 did not react with the non-toxic cereals, suggesting that part of the dodecapeptide is unique to the toxic cereals. However, KG53 did not react with Frazer's fraction III, a physiological peptic-tryptic digest of gluten that is toxic in coeliac disease. This implies that the antibody is recognising an epitope that is cleaved by one of the digestive enzymes pepsin or trypsin. Thus, it seems unlikely that KG53 recognises a toxic amino acid sequence within A-gliadin. The amino acid sequence of the A-gliadin peptide that has homology with the E1b protein of adenovirus 12 is: -Leu-Gly-Gln-Gly-Ser-Phe-Arg-Pro-Ser-Gln-Gln-Asn-. The amine and carboxyl linkages of phenylalanine residues are susceptible to attack by pepsin and it is probable that the action of pepsin, either in vivo or during the preparation of Frazer's fraction III, cleaves the dodecapeptide.

Controversy exists as to the nature of the toxic moiety within gliadin. Analysis of A-gliadin peptides, including amino-acid residues 211–217, has shown that a potentially antigenic β-reverse turn is the predominant structural feature, involving residues -Arg-Pro-Ser-Gln-, which are also present in adenovirus 12.²² Organ culture studies have shown that the tetrapeptide, -Pro-Ser-Gln-Gln-, is found in all coeliac active peptides.²³ Residues 206–217 of A-gliadin

Figure 2: Immunodot assay: results for KG42. Antigens were as follows: (1) unfractionated gliadin, (2) rye, (3) barley, (4) oats, (5) α gliadin, (6) β gliadin, (7) γ gliadin, (8) ω gliadin, (9) maize, (10) millet, (11) sorghum, (12) rice, (13) casein, (14) ovalbumin, (15) Frazer's fraction III, (16) blank.

incorporate this motif; however, in adenovirus 12 the final glutamine is replaced by a cysteine residue. In vitro studies have shown significant reactions to both synthetic A-gliadin and adenovirus 12 peptides from the regions of homology by leukocytes from coeliac patients.^{11 24} However, a recent report using similar methodology, failed to show coeliac activity of a peptide containing the amino acid sequence 211–217 of A-gliadin.²⁵

Infusion of 100 mg of the A-gliadin dodecapeptide caused some, but not significant, histological abnormalities in the jejunal mucosa of two treated coeliac patients.²⁶ However, we have shown that 600 mg of infused unfractionated gliadin produced significant changes in the crypt/villus ratio and epithelial cell height, with a doubling of the intraepithelial lymphocyte count within four hours of the challenge.³ Even assuming that gliadin contains more than one toxic moiety, a pure peptide would contain considerably more of the toxic moiety on a molar basis than whole gliadin. Allowing for intersubject variability, one might still expect to see marked changes in the histology of the small bowel after infusion of 100 mg of pure peptide.

The data presented here suggest that there are sequence homologies between amino acid residues 206–217 of A-gliadin and non-toxic prolamins. Coeliac disease is thought to be a T cell mediated disorder, and since we have used monoclonal antibodies to dissect the antigenicity of these cereal peptides, we will have detected B cell epitopes within the peptides. The possibility therefore exists that there are specific T cell epitopes within the adenovirus 12 E1b protein – A-gliadin sequence homology that are confined to coeliac toxic prolamins. Despite this qualification, we feel that the data presented do not support the hypothesis that prior infection with adenovirus 12 is a precipitating factor in coeliac disease.

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