

Evidence for the role of a human intestinal adenovirus in the pathogenesis of coeliac disease

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SUMMARY We previously noted a region of amino acid sequence homology between A-gliadin, a major α -gliadin component known to activate coeliac disease, and the early region E1b protein of human adenovirus serotype 12 (Ad12), an adenovirus isolated from the human intestinal tract. In the present study sera from coeliac disease patients from the United Kingdom and the United States were assayed for neutralising antibody to Ad12 as evidence of past exposure to that virus and for antibody to synthetic peptides of A-gliadin from the region of shared sequence with the Ad12 E1b protein. Eighty nine per cent of untreated coeliac disease patients had evidence of previous Ad12 infection. There was also a significant increase in the prevalence of neutralising antibody to Ad12 among treated adults (33.3%) and children (30.8%) with coeliac disease compared with controls (0-12.8%) in the western USA and in London. There was no evidence for an increased prevalence of infection with a closely related adenovirus, adenovirus 18, or another enteric virus, Echovirus 11, among coeliac disease subjects. Additional studies documented that a region of A-gliadin that shares amino acid sequence homology with the adenovirus 12 E1b protein could be recognised as an antigenic determinant in active coeliac disease patients. Taken together, these data are compatible with the hypothesis that a viral protein may play a role in the pathogenesis of coeliac disease, perhaps by virtue of immunological cross reactivity between antigenic determinants shared by the viral protein and α -gliadins.

Coeliac disease is characterised by damage to the small intestinal mucosa and the malabsorption of most nutrients. Disease is associated with the dietary ingestion of wheat, rye, barley and oats. Wheat gliadins can be divided into four fractions based on their electrophoretic mobility in polyacrylamide gels; α -, β -, γ -, and ω -gliadins.^{2,3} α -gliadins are known to activate coeliac disease.^{4,5} There is disagreement, however, as to whether γ and ω -gliadins activate disease.^{5,7} A-gliadin has a molecular weight of 31 000 and is a major component of the α -gliadin fraction that is known to activate coeliac disease.⁸⁻¹⁰ The

primary amino acid sequence of A-gliadin has been determined by amino acid sequencing and by the sequencing of a cDNA clone.¹¹

Approximately 25% of monozygotic twins and 70% of serologically HLA identical siblings are discordant for coeliac disease despite the ingestion of similar dietary grains.¹²⁻¹⁴ Thus, we proposed that other environmental factors may be important in the pathogenesis of coeliac disease.¹⁵ In this respect, we reported a region of amino acid sequence homology between A-gliadin, and the early region E1b protein of human adenovirus serotype 12 (Ad12),¹⁵ an adenovirus usually isolated from the human intestinal tract and stool specimens.¹⁶⁻¹⁸ The E1b protein is a non-structural protein encoded by the Ad12 genome that is expressed early in lytic infection

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with Ad12, and is the predominant cytoplasmic protein expressed in mammalian cell lines that are transformed by Ad12.¹⁵⁻¹⁶

The above observations suggested the possibility that molecular mimicry may be important in the pathogenesis of coeliac disease by virtue of immunologic cross reactivity between a virus encoded protein and a peptide fragment of gliadin.¹⁵ The present study provides evidence of previous exposure to adenovirus 12 among coeliac disease patients and indicates that the region of A-gliadin that has amino acid sequence homology with the Ad12 E1b protein can serve as an antigenic determinant.

Methods

SUBJECTS

Sera were obtained from the following subject groups: (a) London, England: 18 adult subjects with active untreated coeliac disease, 19 adult subjects with coeliac disease in remission after treatment with a gluten free diet, and 35 controls from the same institution having gastrointestinal disorders other than coeliac disease. The latter included 23 with irritable bowel syndrome, and the remainder with peptic ulcer disease, oesophagitis and lactose intolerance; (b) San Diego County, USA: 30 adult subjects with coeliac disease in remission after treatment with a gluten free diet. Control sera for this group were 30 ethnic, age and sex matched healthy individuals in San Diego County; (c) London, England: 13 children (ages 9 months to 14 years) with coeliac disease in remission on a gluten free diet. Controls included 70 children from the same institution. Of the controls, 10 had cow's milk protein or other food intolerances, 12 had inflammatory bowel disease (Crohn's disease or ulcerative colitis), and the remainder had other gastrointestinal complaints – for example, diarrhoeal illnesses, giardiasis, Bechets syndrome, or failure to thrive. Subjects with treated coeliac disease had been maintained on a gluten free diet for a minimum of six months, and in some cases as long as 20 years, before study.

VIRUS NEUTRALISATION TEST

Microneutralisation tests were done in flat bottom Costar 96-well tissue culture plates (Costar, Cambridge, MA) in Dulbecco's modified eagle medium supplemented with 2% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. Sera were tested using serial two fold dilutions and a challenge dose of 16 TCID₅₀ of Ad12 (strain Huie; ATCC VR-863, American Type Tissue Culture Collection, Rockville, MD) or Ad18 (ATCC VR 1095) and 100 TCID₅₀ of Echovirus 11 (ATCC VR 1052) as determined by

back titration. Tissue culture plates with test serum and virus were incubated at room temperature (RT) for one hour, after which 10⁶ A549 human lung carcinoma cells (CCL184, American Type Tissue Culture Collection) in 50 µl were added to each well. To determine the neutralisation endpoint, wells were examined for cytopathic effects after five to seven days incubation. Each assay contained A549 cells with no added virus and A549 cells with no added serum as negative controls. Wells contained A549 cells with virus and serial dilutions of rabbit or equine antiserum to the respective viruses as positive controls. Rabbit antiserum to Ad12 (ATCC VR-1089 AS/Rab) or Ad18 (ATCC VR-1095 AS/Rab), and equine antiserum to Echovirus 11 (ATCC VR-1052 AS/Ho) were obtained from the American Type Tissue Culture Collection (Rockville, MD). All human test sera were coded before testing and neutralisation studies and neutralisation endpoints were conducted and read by a single individual blinded as to the code. Reciprocal neutralising antibody titres greater than 8 arbitrarily were regarded as positive.

PREPARATION OF WHOLE GLIADIN AND A-GLIADIN

Whole gliadin derived from unbleached flour from bread wheat variety Scout 66 was prepared as described previously.¹⁹ Polyacrylamide gel electrophoresis in pH 3.2 aluminium lactate buffer showed no evidence of albumins moving faster than gliadins, indicating the preparative approach was effective in separating gliadins from albumins.²⁰

To prepare A-gliadin, 'Scout 66' unbleached flour was extracted with 0.01 M acetic acid at 35°C and centrifuged to remove insoluble material. The extract was ultracentrifuged, after which the pellet was solubilised, aggregated, and ultracentrifuged, and the solubilisation, aggregation and ultracentrifugation were repeated once more. The resulting pellet was solubilised in low pH buffer, chromatographed on Bio-Gel P-60 in 0.06 M ammonium formate (pH 3.1) and lyophilised.

PEPTIDE SYNTHESIS

A 6 residue peptide (6 mer) having A-gliadin residues, 212–217 – that is, RPSQQN, and a 12 residue peptide (12 mer) having A-gliadin residues, 206–217 – that is, LGQGSFRPSQQN, were prepared by solid phase peptide synthesis.²¹⁻²³ A 10 residue peptide of cytochrome C (10 mer) – that is, CH₃CO.CGDVEGYLKK, was used as a control peptide. The amino acid compositions of the peptides were determined by amino acid analysis and conformed to expected values.

RADIOIMMUNOASSAY (RIA) FOR ANTIGLIADIN ANTIBODY

Serum antibody to whole gliadin, A-gliadin or synthetic gliadin peptides was measured as previously described.²⁰ Briefly, 96-well Flexivynyl U-bottom microtitre plates were coated with whole gliadin, A-gliadin, or synthetic A-gliadin peptides coupled to bovine serum albumin (BSA) by established procedures using glutaraldehyde.¹⁵ Control plates were coated with 1% BSA and all plates were quenched with 1% BSA. In experiments to test the ability of synthetic peptides to inhibit the binding of antibody to gliadin coated wells, 25 μ l titrated concentrations of synthetic peptide were added to wells before the addition of test serum. Specific binding was obtained by subtracting background counts in BSA-coated wells from counts in gliadin or synthetic peptide coated wells. All assays were done in triplicate.

STATISTICAL ANALYSIS

The statistical significance of differences in the prevalence of virus neutralising antibody titres between coeliac disease patients and controls was calculated using χ^2 test. Student's *t* test was used to determine the significance of differences between antigliadin and antigliadin peptide antibody concentrations, and the significance of peptide inhibition in the radioimmunoassay in coeliac disease patients and controls.

Results

Neutralising antibody to Ad12 is directed to determinants on the structural hexon (ϵ) protein of Ad12, a protein that is not related to the Ad12 E1b protein that shares amino acid sequence homology with A-gliadin.²⁴ Thus, in this study, neutralising antibody to Ad12 was measured strictly as an indicator of previous Ad12 exposure. As shown in Figure 1, 89% (16/18) of the group of untreated coeliac disease patients in London had neutralising antibody to Ad12. In contrast, such antibody was present in only 5% (1/19) of treated coeliac disease subjects on a gluten free diet, and in 17% (6/35) of disease controls from the same institution (Fig. 1). Titres for individual subjects are shown in Table 1. There was no significant difference in the prevalence of neutralising antibody to Echovirus 11 among individuals with untreated or treated coeliac disease, or disease controls (Fig. 1). By comparison, 33.3% (10/30) of treated coeliac disease patients in San Diego County, but none of a group of matched healthy controls had Ad12 neutralising antibody in their serum ($p < 0.05$) (Table 2). Neutralising antibody to Ad12 was present in 30.8% (4/13) of children in London, England with

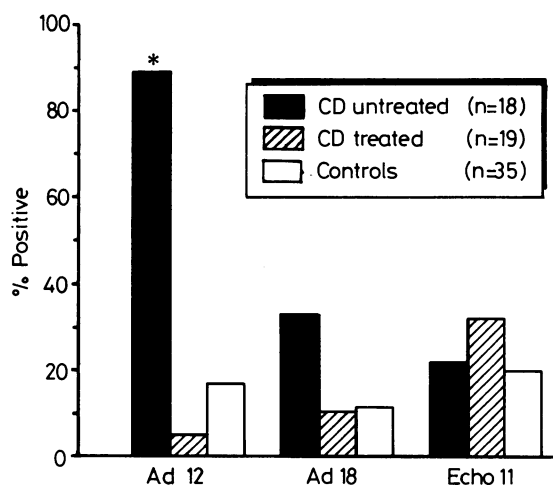


Fig. 1 Adenovirus and Echovirus neutralising antibody among untreated and treated adult coeliac disease (CD) subjects and controls in London. *n* = number of subjects in each group. * indicates value significantly different from control group ($p < 0.05$).

coeliac disease on a gluten free diet compared with 12.8% (9/70) of disease controls ($p < 0.05$).

Antibody to Ad18 in coeliac disease patients was specific for that serotype. There was no significant difference in the presence of neutralising antibody to adenovirus type 18, an adenovirus closely related to Ad12, between coeliac disease subjects and controls (Fig. 1, Table 1). Antibody to Ad18 was present in 33% (6/18) of untreated adult coeliac disease subjects in London compared with 17% (6/35) controls (Fig. 1). These data, however, did not achieve significance and, as shown in Table 1, titres to Ad18 exceeded those to Ad12 in 2 of the untreated adult coeliac subjects whereas in the remaining four subjects, titres to Ad12 exceeded those to Ad18.

Antibody to whole gliadin, A-gliadin as well as a 6 amino acid synthetic peptide RPSQON of A-gliadin (6 mer) from within the region of homology between A-gliadin and the Ad12 E1b protein was present in the serum of untreated coeliac disease patients in London (Fig. 2). The same individuals did not have measurable antibody to an irrelevant control synthetic 10 amino acid peptide of cytochrome C by RIA. Although antibody to a 12 amino acid peptide of A-gliadin encompassing the region of homology with the Ad12 E1b protein was not detected in binding studies, the presence of such antibody in patient sera could be shown in inhibition studies. Thus, approximately 20% of the serum antibody binding to epitopes on the intact A-gliadin molecule – that is, molecular weight of 31 000, could be inhibited by either the synthetic 6 or 12 amino acid A-gliadin

Table 1 Reciprocal neutralising antibody titres to Ad12 and Ad18 in untreated and treated coeliac disease subjects and controls

Untreated		Treated			Controls			
Subject no.	Ad12	Ad18	Subject No.	Ad12	Ad18	Subject Ad12 No.	Ad18	
1	128	16	1	16	<2	1	32	32
2	128	4	2	8	<2	2	32	16
3	64	<2	3	4	<2	3	32	<2
4	64	<2	4	4	<2	4	16	16
5	64	<2	5	2	16	5	16	16
6	32	4	6	2	2	6	16	8
7	32	8	7	2	2	7	8	16
8	32	16	8	2	<2	8	8	16
9	32	16	9	2	<2	9	8	8
10	32	16	10	2	<2	10	4	4
11	32	4	11	2	<2	11	4	2
12	32	4	12	<2	16	12	4	4
13	16	2	13	<2	2	13	4	<2
14	16	2	14	<2	2	14	4	4
15	16	<2	15	<2	<2	16	2	2
16	16	<2	16	<2	<2	16	2	2
17	8	16	17	<2	<2	17	2	2
18	4	16	18	<2	<2	18	2	2
			19	<2	<2	19	2	2
						20	2	2
						21	2	<2
						22	2	<2
						23	2	<2
						24	2	<2
						25	2	<2
						26	<2	<2
						27	<2	2
						28	<2	2
						29	<2	<2
						30	<2	<2
						31	<2	<2
						32	<2	<2
						33	<2	<2
						34	<2	<2
						35	<2	<2

peptide (Fig. 3), but not by the control 10 amino acid cytochrome C peptide. In contrast, treated adult coeliac disease patients from London had detectable serum antibody to whole gliadin and A-gliadin but did not have antibody, as detected in binding (Fig. 2) or inhibition radioimmunoassays, to peptides from the region shared by A-gliadin and the Ad12 E1b protein. In addition, none of the sera from the treated adult coeliac disease patients in San Diego County or the treated coeliac disease children from London reacted with the synthetic gliadin peptides although, as previously reported, serum from some of those individuals variably reacted with whole or A-gliadin.^{20,25}

Discussion

We postulated that Ad12 may play a role in the pathogenesis of coeliac disease by virtue of chance

Table 2 Adenovirus and echo virus neutralising antibody among coeliac disease and control populations in the US and the UK

Subject source	Subjects tested (n)	% (number) of subjects with reciprocal titres > 8	
		Adenovirus type 12	Adenovirus type 18
San Diego:			
Coeliac disease	30	33.3% (10)*	3.3% (1)‡
Controls	30	0.0% (0)	3.3% (1)
London (children):			
Coeliac disease	13	30.8% (4)†	8.3% (1)‡
Controls	70	12.8% (9)	4.3% (3)

* Value in patient group significantly different from control group ($\chi^2 = 11.64$; $p < 0.05$). Reciprocal titres in all controls were 2 or < 2; † Value in patient groups significantly different from control group ($\chi^2 = 3.88$; $p < 0.05$). Positive controls included 1/12 children with inflammatory bowel disease (titre = 16) and 1/20 children with cow's milk protein and other food intolerances (titre = 16); ‡ Value in patient group not significantly different from control group.

immunologic cross reactivity between shared epitopes on an Ad12 viral encoded protein and a gliadin peptide. If this is the case, one would predict that coeliac disease patients would have evidence of past exposure to Ad12. In this study, 89% of a group of untreated coeliac disease patients, had evidence of previous infection with human adenovirus serotype 12. Neutralising antibody titres to Ad12 were significantly increased also in adults and children with coeliac disease maintained long term on a gluten free diet compared with healthy or disease controls. The greater prevalence of neutralising antibody to Ad12 in untreated than treated coeliac disease patients may

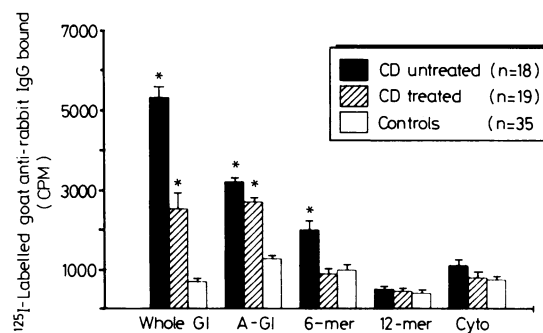


Fig. 2 Solid phase radioimmunoassay for antibody to whole gliadin, A-gliadin and synthetic 6 mer and 12 mer A-gliadin peptides in untreated and treated adult coeliac disease (CD) subjects and controls in London. n = number of subjects in each group. Values are means and bars represent \pm SEM. Asterisks indicate values significantly different from control group ($p < 0.05$). Cyto is a 10 residue synthetic peptide of cytochrome c.

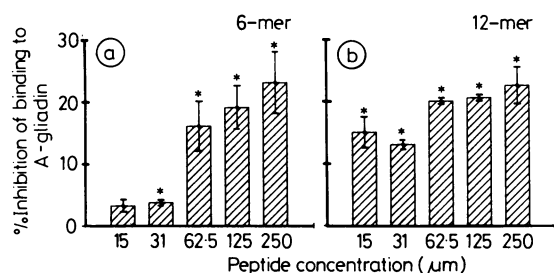


Fig. 3 Ability of 6 mer (A) and 12 mer (B) synthetic gliadin peptides to inhibit antibody binding to A-gliadin. Sera from eight adult patients with untreated coeliac disease were assayed for anti-A-gliadin antibody by RIA in the presence of varying concentrations of 6 mer or 12 mer or in the absence of synthetic peptide as described in methods. Percent inhibition of binding to A-gliadin =

$$1 - \frac{^{125}\text{I-labelled goat anti-rabbit IgG bound in presence of peptide}}{^{125}\text{I-labelled goat anti-rabbit IgG bound in absence of peptide}} \times 100$$

A control cytochrome c decapeptide tested at the identical concentrations resulted in no inhibition of antibody binding to A-gliadin. Values are means and bars represent \pm SEM. Asterisk indicates binding to A-gliadin was significantly inhibited in the presence of peptide ($p < 0.05$).

reflect more recent infection with Ad12, although evidence in the present study does not directly address this issue. The prevalence of antibody to Ad12 in adult control patients and in children with intestinal disorders other than coeliac disease approximated the 0–15% prevalence noted before by others for Ad12 neutralising antibody among different control populations²⁶ (Hierholzer, JC personal communication).

Little epidemiologic data are available regarding Ad12, although such double stranded DNA viruses can be detected in the human intestinal tract as early as the first one to two years of life.^{16, 26} Adenovirus 12 has not been implicated previously as a cause of human disease, and has been studied in large measure because of its known ability to transform mammalian cell lines.^{16, 27} The transforming activity of Ad12 has been assigned to the left hand 11% of the viral genome – that is, early region I,²⁹ which contains two transcriptional units, E1a and E1b in a 3.9 kb DNA segment.^{30, 31} Those units are the first to be expressed during lytic infection of cells. The E1b genomic region codes for the 54-kD protein that shares a region of sequence homology with A-gliadin, as well as a 19-kD polypeptide.^{32, 33}

Antibody to Ad12 in coeliac disease subjects did not appear to reflect a general increase in antibody titres to multiple viruses in those individuals. Thus, the prevalence of serum antibody to Ad18, a virus closely related to Ad12, and to Echo 11, another human intestinal virus, did not differ significantly

between the coeliac disease patients and controls. The finding of antibody to Ad18 in 6/18 untreated coeliac disease patients in London appears to reflect the presence of antibody to Ad18 in those patients and not serologic cross reactivity between Ad12 and Ad18.³⁴ Moreover, the presence of Ad18 neutralising antibody in 3–13% of the control populations in this study approximates the previously reported prevalence of such antibody in 5–20% of individuals in control populations.²⁶ We note that the present study does not totally exclude the possibility that independent of a role in disease pathogenesis coeliac disease patients, perhaps by virtue of abnormalities in their epithelium and mucosa, may be more likely than controls to acquire adenovirus 12 infection.

Serum antibody from untreated coeliac disease patients reacted in the solid phase radioimmunoassay with a 6 residue peptide but not a 12 residue peptide from the region of shared amino acid sequence between A-gliadin and the Ad12 E1b protein, whereas both the 6 and 12 residue peptides could inhibit the binding of patient antibody to the intact A-gliadin molecule. The apparent differences in reactivities of the 6 and 12 residue peptides in the solid phase RIA, compared with the ability of both peptides to inhibit binding of serum antibody to A-gliadin, likely reflects differences between the assays in the way in which antigen is presented to the antibody. In the solid phase radioimmunoassay peptides conjugated to bovine serum albumin are used to coat polyvinylchloride plates, whereas in the competition assays peptides are free in solution. These two conditions may result in different conformational constraints on the peptide or in different degrees of exposure of residues important for antibody binding.³⁵

The data in this study document an increased prevalence of past infection with Ad12 among coeliac disease patients and establish that a region of A-gliadin that has amino acid sequence homology with an adenovirus E1b protein can act as a determinant for antibody recognition. These data are consistent with a hypothesis that shared epitopes between a protein coded for by a virus and α -gliadins may play a role in the pathogenesis of coeliac disease by virtue of molecular mimicry, either at the level of T or B cell recognition. A striking feature of coeliac disease is its association with the serologically defined HLA-class II molecules -DR3, -DR7 and -DQw2,^{1, 36, 37} and a 4 kb HLA-D region β chain genomic restriction fragment length polymorphism.³⁸ Processed antigen in the form of peptide fragments, in conjunction with such genetically encoded HLA class II molecules on antigen presenting cells, functionally form a bimolecular complex that is recognised by the receptor for antigen on helper T cells.³⁹ The strong

association between coeliac disease and specific class II HLA molecules, taken together with the present data, suggests that peptides shared by A-gliadin and a viral encoded protein may cross react also at the level of recognition by T helper cells. Such T cells could help in the induction of antibody responses to antigenic determinants in the region of shared sequence, as detected in this study, as well as to determinants on other parts of the α -gliadin molecule. Further, if the region of homology between A-gliadin and the Ad12 E1b protein acts as a T helper cell determinant, antibody to epitopes on α -gliadins that cross react with γ and ω -gliadins and barley or rye prolamins would be predicted.

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