

Is persistent adenovirus 12 infection involved in coeliac disease? A search for viral DNA using the polymerase chain reaction

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Abstract

It has been shown that partial amino acid sequence homology between α gliadin and an early region protein (E1B-58 kDa) of adenovirus 12 results in immunological cross reaction. This led to the proposal that prior infection by adenovirus 12 could be associated with the development of coeliac disease. To examine this hypothesis, evidence was sought of persistent adenovirus 12 infection in the small intestinal mucosa of patients with coeliac disease. DNA isolated from biopsy samples from 24 control and 18 coeliac disease patients was analysed by the polymerase chain reaction for adenovirus 12 DNA encoding the E1B-58 kDa protein. Four of 18 coeliac disease and two of 24 control patients were positive. There is thus a low prevalence of this infection on both groups of patients but certainly no significantly increased incidence in coeliac disease. These results suggest that persistent

adenovirus 12 infection is not a major element in the pathogenesis of coeliac disease.

There has been considerable interest in the hypothesis that prior infection with adenovirus 12 may lead to the development of coeliac disease in susceptible individuals on exposure to dietary gliadin. This was proposed because a degree of sequence homology was noted between amino acid residues 384-395 of the E1B-58 kDa protein of adenovirus 12 and amino acid residues 206-217 of α gliadin and immunological cross reactivity between these two peptides was shown.¹ It was therefore suggested that this cross reactivity might be the basis of the pathogenesis of the coeliac lesion. In support of this hypothesis, neutralising antibody titres to the viral hexon protein were found to be raised in coeliac patients,² suggesting that coeliac patients had an increased prevalence of adenovirus 12 infection. The antibody measured in these studies, however, was directed against coat proteins of the virus and not against the E1B-58 kDa protein. Kagnoff³ has suggested that cross reactivity between gliadin and adenovirus 12 E1B-58 kDa is at the level of T cell recognition and that such cross priming of T cells leads to the generation of the helper T cell response and antibody production.

In further support of the proposed mechanism, treated coeliac disease patients have a cell mediated immune response in the peripheral blood to a synthetic gliadin peptide analogous to the E1B-58 kDa viral protein,⁴ and recent studies have shown similar cellular hypersensitivity to a synthetic viral peptide of the homologous sequence.⁵ There is, however, no evidence yet available of T cell reactivity to the E1B-58 kDa protein itself and we failed to show specific antibodies to this particular protein in coeliac disease patients.⁶ This raises some doubts therefore about the hypothesis.

A different approach to this hypothesis would be to seek evidence of persistent adenovirus 12 infection in patients with coeliac disease. Persistent infection would require continuing viral replication, and since the early region proteins (E1A and E1B) are involved in the initiation of replication, one would expect to find evidence of the early protein genes in a persistent infection. Hence we have used the polymerase chain reaction to seek evidence of persisting viral infection in small intestinal mucosa using specific oligonucleotide primers for the E1B-58 kDa gene. This is particularly appropriate since it is the E1B-58 kDa protein that is involved in the original hypothesis.

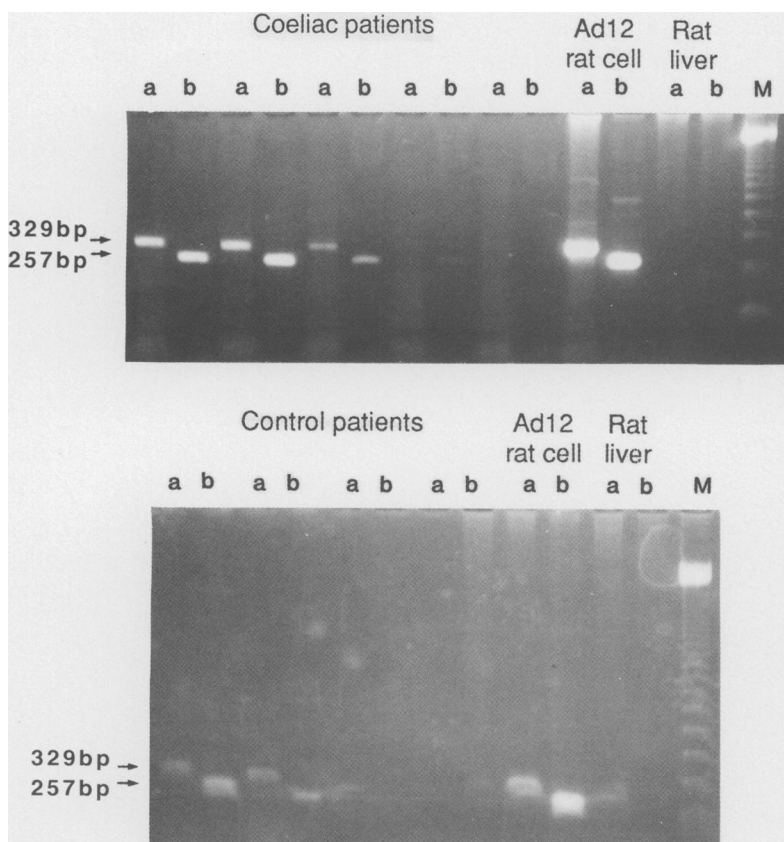


Figure 1: Agarose gel electrophoretic analysis of polymerase chain reaction (PCR) products. PCR products using adenovirus (Ad) 12 E1B primers #1 and #2 (tracks a) or #1 and #3 (tracks b) and jejunal biopsy specimen DNA from coeliac disease (above) or control patients (below) were analysed. M = 123 bp molecular weight 'ladder'.

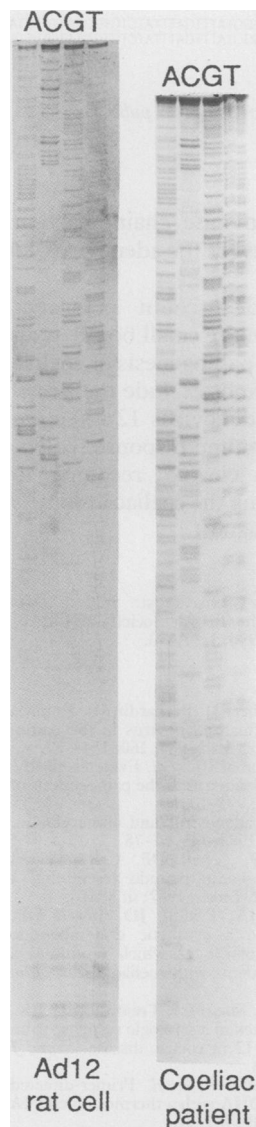


Figure 2: DNA sequencing of the 329 bp polymerase chain reaction product.

Patients and methods

Upper small intestinal mucosal biopsy specimens were taken from 24 control and 18 coeliac disease patients. Twelve of the control patients were men; the mean age was 41 years (range 9-70). Intestinal biopsy was performed for the routine investigation of diarrhoea (9), dyspepsia (7), anaemia (5), short stature (1), weight loss (1), and lymphangiectasia (1). All biopsy specimens had normal histological appearances, except for variable dilated lymphatics in the patient with lymphangiectasia.

Ten of the 18 coeliac disease patients were men; the mean age was 49 years (range 23-73). Eleven patients have been treated with a gluten free diet for at least one year (mean 5-6 years, range 1-20). All had shown a good clinical and histological response to the gluten free diet. Seven patients were newly diagnosed and had the typical histological features of untreated coeliac disease. They have yet to undergo further biopsy to assess the effect of a gluten free diet. This study was approved by the local ethical committee.

The biopsy specimens were immediately stored frozen at -70°C until processing was performed (maximum storage time two months). They were homogenised in acid washed glass homogenisers with 0.1 ml 10 mmol/l Tris-HCl (pH 8.0), 1 mmol/l EDTA. Further homogenisation was performed after the addition of 1.0 ml lysis buffer (0.5% Sarkosyl, 0.5 mol/l EDTA), proteinase K was added to the pooled mixture to a final concentration of 150 $\mu\text{g}/\text{ml}$, and it was incubated for 18 hours at 37°C . Samples were then extracted with phenol and dialysed overnight at 4°C in TEN buffer (50 mmol/l Tris-HCl pH 8.0, 10 mmol/l NaCl, 10 mmol/l EDTA). Polymerase chain reaction analysis was performed (in 50 μl reactions) using 100 ng genomic DNA, 1 $\mu\text{mol}/\text{l}$ oligonucleotide primers, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l MgCl_2 , 0.1% Triton X-100, 0.01% gelatin, 0.2 mmol/l each dATP, dCTP, dGTP, and dTTP (Pharmacia) and 2.5 units Taq polymerase (Promega). Reactions were overlaid with 20 μl light mineral oil and denatured for seven minutes at 95°C . They were then subjected to 30 cycles of the following programme in a thermocycler (Perkin Elmer Cetus): 60°C , one minute; 72°C , one and a half minutes; 95°C , one minute. An aliquot (15 μl) of the reaction products was analysed by electrophoresis on a 2% agarose gel containing 10 $\mu\text{g}/\text{ml}$ ethidium bromide.

The following oligonucleotides were used (with their positions given on the adenovirus 12 DNA sequence):

Ad12 E1B#15' > CTGTTTCATATTGTGAGCCAC > 3' (NT 2912-2931)
 Ad12 E1B#25' > AAGGTGGTCACCTCTCAGCT > 3' (NT 3240-3221)
 Ad12 E1B#35' > CTAGATGACTGCTACCCAC > 3' (NT 3187-3168)

Primers #1 and #2 generated a 329 bp polymerase chain reaction product and #1 and #3

gave a 257 bp species. Both polymerase chain reaction products span the sequence in the adenovirus 12 E1B-58 kDa gene which encodes the peptide sequence homologous to A-gliadin.

The polymerase chain reaction products using primers #1 and #2 were sequenced by concentration and purification of amplified DNA using an Ultrafree MC microfiltration unit (Millipore) and following the manufacturer's instructions. Approximately 750 ng of double stranded DNA was sequenced using a T7 sequencing kit (Pharmacia) with primer #1 and [^{35}S]dATP. Sequencing products were separated by electrophoresis on 6% denaturing polyacrylamide gels followed by autoradiography.

Results

Synthetic oligodeoxynucleotides specific for the adenovirus 12 E1B-58 kDa coding region were used in polymerase chain reaction amplification of adenovirus 12 sequences in DNA isolated from jejunal biopsy specimens from coeliac disease or control patients. DNA from an adenovirus 12 transformed rat cell line (which contains integrated adenovirus 12 DNA sequences) served as a positive control, while rat liver DNA was used as a negative control. Figure 1 shows that primers #1 and #2 (tracks a) yielded a 329 bp product using DNA of three coeliac disease and two control patients, while primers #1 and #3 gave the expected 257 bp species (tracks b) from the same patients. The 329 and 257 bp fragments generated from patient material exactly comigrated with the polymerase chain reaction products from the adenovirus 12 transformed cell DNA, strongly suggesting that these polymerase chain reaction products had been amplified from adenovirus 12 DNA sequences. Control rat liver DNA did not generate either polymerase chain reaction product. Restriction endonuclease analysis of the patient's polymerase chain reaction products using the enzymes Taq I or RsaI gave fragments with sizes expected from the published adenovirus 12 E1B DNA sequence* (results not shown). To prove conclusively that the 329 bp polymerase chain reaction product was indeed derived from adenovirus 12 DNA, the double stranded product was subjected to DNA sequencing by the dideoxy method. The partial DNA sequence of the polymerase chain reaction product of a coeliac disease patient was identical to that of the 329 bp product from the adenovirus 12 transformed rat cell (Fig 2). The partial DNA sequences of the polymerase chain reaction products from coeliac disease and control patients and the adenovirus 12 transformed rat cell were identical to the published E1B sequence of adenovirus 12 viral DNA (Fig 3).⁸ This conclusively establishes the identity of the polymerase chain reaction products from the coeliac disease and control patient samples as adenovirus 12 E1B DNA.

Although adenovirus 12 E1B DNA sequences could be detected in the mucosa of some coeliac disease patients, most did not contain amplifiable E1B DNA sequences (Table). An approximately similar proportion of control patients also contained adenovirus 12 E1B DNA. There is no statistically significant difference between these

Prevalence of adenovirus (Ad) 12 DNA in the small bowel mucosa of coeliac disease and control patients

	Coeliac	Control
Positive for Ad 12 DNA	4	2
Negative for Ad 12 DNA	14	22

Ad12 viral DNA TGTACCATACATTTAGGC Ad12 rat cells Control patient Coeliac patient TGTACCATACATTTAGGC	TTAAGCGGGGTATGTTTAGACCTTCCAATGTAAC GGCGGG-TA-GTTTAGACCTTCCAATGTAAC TTA-GGCGGGGTATGTTTAGACCTTCCAATGTAAC TTAAGCGGGGTATGTTTAGACCTTCCAATGTA-C	TTCAGCCACTCAAAACATTATGCTGGAACCTGAAGTGTTTTCTAGAGTGTGTTTAAATGGGGTATTTGATTATCTGTGGAATATTGTA TTCAGCCACTCAAAACATTATGCTGGAACCTGAAGTGTTT-CTAGAGTGTGTTTAAATGGGGTATTTGATTATCTGTGGAAT-ATG-A TTCAGCCACTCAAAACATTATGCTGGG-A-CTGAAGTGTTT TTCAGCCACTCAAAACATTATGCTGGAA-CTGAAGTGTTT-CT
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Figure 3: DNA sequences of coeliac, control, and adenovirus (Ad) 12 transformed rat cell polymerase chain reaction samples compared with the published sequence⁸ of Ad12 viral DNA. The sequence encoding the region of Ad12 E1B-58 kDa homologous to α gliadin is boxed.

two groups of patients. For example, two of the five coeliac disease patients tested in Figure 1 did not show amplifiable E1B DNA sequences with either combination of primers. Reamplification of polymerase chain reaction products has been reported to increase the sensitivity of the assay,⁹ but this did not generate adenovirus 12 E1B polymerase chain reaction products in samples that were negative in the first polymerase chain reaction (not shown). To confirm that all DNA samples tested were amplifiable, the polymerase chain reaction was performed using primers to the human p53 antioncogene. All DNA samples were amplified using these primers (not shown). Thus, although a proportion of coeliac disease patients contain adenovirus 12 E1B DNA in the intestinal mucosa, this does not correlate with the incidence of the disease and may reflect the general incidence of adenovirus 12 infection in patients with gastrointestinal disorders. Even among those coeliac disease patients whose intestinal mucosa did contain adenovirus 12 E1B DNA, there was no correlation with stage of the disease – that is whether treated or untreated.

Discussion

This study has shown that there is no clear association between the presence of adenovirus 12 DNA in the small bowel mucosa and coeliac disease, at any stage of treatment. A previous study, using Southern blotting and nucleic acid hybridisation techniques, failed to detect adenovirus 12 or 41 viral DNA in duodenal biopsy samples from coeliac disease patients at a sensitivity of one copy of viral DNA per cell.¹⁰ Our findings differ from this previous study in that adenovirus 12 E1B DNA could be detected in the jejunal mucosa of some coeliac disease patients, but that this situation was also true for a similar proportion of control patients. The much greater sensitivity of the polymerase chain reaction assay used here probably explains these differences. We have not yet determined whether other regions of the adenovirus 12 viral genome are present in the mucosa of the patients described here, although this could also be

determined by the polymerase chain reaction using published sequences for the adenovirus 12 E2 and E4 genes.^{11,12}

Our results therefore discount persistent adenovirus 12 infection in the small bowel as an important element in the pathogenesis of coeliac disease. However, we cannot exclude the possibility that an initiating adenovirus 12 infection may trigger a cellular immune response which, after the virus has been cleared, responds to dietary α gliadin by eliciting the coeliac lesion, as originally proposed by Kagnoff.¹

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