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DETERMINATION OF ANTIBODIES TO NON-STRUCTURAL AND STRUCTURAL ADENOVIRUS ANTIGENS BY ELISA

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Summary. – We evaluated possibilities to analyze serum antibodies to non-structural (peptides derived from adenovirus E1b protein) and structural (hexon) adenovirus antigens by ELISA. Synthetic dodecapeptides covering a putative A-gliadin cross-reactive antigenic determinant of the E1b protein were used. The aminoterminus of the peptides appeared to be important for antibody binding but the exact sequence of a possible common B-cell epitope within the peptides remained open. Coupling of the peptides to a carrier protein was essential for ELISA analyses of serum antipeptide antibodies. IgA antibodies to both adenovirus derived E1b peptides and hexon antigen could be detected already two weeks after the onset of an acute adenovirus infection, while antipeptide IgG antibodies were seen in a restricted number of patients only.

Key words: adenovirus; antibodies; coeliac disease; early antigen; gliadin; synthetic peptides

Introduction

Adenovirus type 12 (Ad12) is associated to coeliac disease CD by seroepidemiological studies (Kagnoff *et al.*, 1987; Arato *et al.*, 1991). So far direct evidence of a persistent adenovirus infection in the gut of patients with CD is equivocal (Carter *et al.*, 1989; Mahon *et al.*, 1991). New tools for the study of adenovirus-coeliac disease association are urgently needed.

A-gliadin is the toxic fraction of gluten and is known to activate CD (Howdle *et al.*, 1984). There is considerable amino acid homology within a 12 amino acid long residue (**L R R G M F R P S Q C N**) of human adenovirus type 12 (Ad12) E1b protein and A-gliadin (**L G Q G S F R P S Q Q N**) (Kagnoff *et al.*, 1984). Antibodies to a 6 amino acid long synthetic peptide (R P S Q Q N) derived from A-gliadin have been found in the sera of untreated CD-patients (Kagnoff *et al.*, 1987). CD-patients also show abnormal cell-mediated immune responses, e. g. delayed hypersensitivity reactions to the 12 amino acid long

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A-gliadin peptide (Karagiannis *et al.*, 1987; Mantzaris *et al.*, 1990). However, analysis of serum antibody responses to Ad12 E1b derived peptide or corresponding peptides stemming from other adenovirus types has not been performed. We have evaluated different adenovirus and A-gliadin peptides for antibody analysis and compared the results with adenovirus common hexon antigen.

Materials and Methods

Patients. Convalescent phase serum samples from 16 children hospitalized for an acute adenovirus infection (8 girls and 8 boys, mean age 6.7 years, range 1.1 - 13.3 years) were available from the Department of Clinical Microbiology of Tampere University Central Hospital. The adenovirus diagnoses were made by determining IgM class antibodies against adenovirus hexon antigen by ELISA (Waris and Halonen, 1987) or demonstrating a fourfold rise between acute and convalescent phase serum samples in routine complement fixation test. We have chosen one adenovirus seropositive and one seronegative age-matched healthy control from children attending the hospital for elective surgigal operations. Reference positive sera were chosen from patients with adenovirus seroconversion in both the ELISA and the CF-test, or patients who were positive for anti-gliadin antibodies (Savilahti *et al.*, 1983).

Synthetic peptides. The Ad12 E1b derived peptide (L R R G M F R P S Q C N) was originally described by Kagnoff *et al.* (1984). Open reading frame (ORF) E1b of an enteric adenovirus type 40 (Ad40, Ishino *et al.*, 1988) was compared to that of Ad12 (Kimura *et al.*, 1981) in order to identify a similar peptide. A twelve amino acid long peptide (A R R G M F S P Y Q S N) was chosen for further analyses. In addition, a corresponding peptide from A-gliadin (L G Q G S F R P S Q Q N) was chosen. The peptides containing an additional carboxyterminal cysteine were purchased from Cambridge Research Biochemicals Ltd (CRB, Cambridge).

We also synthesized corresponding peptides according to Geysen *et al.* (1987) using an Epitope Scanning kit (CRB). In the Geysen method peptides become attached on polyethylene rods from their carboxyterminal end. The rods are assembled in a microtiter plate format to facilitate their later use in ELISA. Fmoc/t-butyl protected amino acids (CRB) and high quality dimethylformamide (DMF) (Applied Biosystems, Warrington) were used as previously described (Lehtinen *et al.*, 1990).

Conjugation of peptides. The peptides were conjugated to keyhole limpet haemocyanin (KLH) (Sigma, St. Louis) from their carboxyterminal end by using maleimidobenzoic acid N-hydroxysuccimide ester (MBS, Sigma) conjugation according to Neurath *et al.* (1982). First 8 mg of KLH was dissolved into 1 ml of 10 mmol/l phosphate buffer (pH 7.4) and 2 mg MBS was dissolved in 300 µl of DMF. KLH was activated by mixing it with 160 µl of MBS. Activated KLH was centrifuged (3000 rpm, 15 min) and the supernatant was collected and loaded on a Sephadex G-25 column (Pharmacia, Uppsala). The eluate was collected in 0.7 ml fractions and the protein-rich fractions were pooled. Next, 1.5 ml of activated KLH 0.5 ml of peptide solution (5 mg peptide disolved into 500 µl of 50 mmol/l phosphate buffer, pH 6.0), 0.7 ml of distilled water and 0.3 ml of 10 mmol/l phosphate buffer were mixed by continuous stirring for 3 hr at RT. Finally, the conjugate was filtered through a 450 nm filter (Millipore, Bedford) and stored at -20 °C. Conjugation of the peptides from the aminoterminal end was done by using glutaraldehyde as described by Szelke (1983). First, 4 mg of KLH and 5 mg of peptide were separately dissolved into 0.3 ml and 0.5 ml 0.1 mol/l sodium hydrogen carbonate buffer (buffer A, pH 8.4) by continuous stirring for 1 hr. Next, the KLH solution, the peptide solution, 10 µl of glutaraldehyde and 1.2 ml of buffer A were mixed by vigorous shaking in ice for 3 hr. The conjugate was dialyzed against 0.9 % NaCl solution for 48 hr, stored at -20 °C and filtered through a 450 nm filter before use.

ELISA for non-structural adenovirus antibodies. Conjugated and unconjugated peptides were used as antigens in ELISA. KLH was used as a control antigen. Nunc Macrosorb^R microtiter plates (Nunclon, Århus) were coated with an antigen concentration of 20 µg/well by using 0.1 mol/l carbonate buffer (pH 9.5). After an overnight incubation the plates were washed three times with

PBS + 0.5 % Tween 20 and three times with distilled water. The plates were saturated with 10 % FCS in PBS (pH 7.2) for 1 hr at 37 °C and immediately reacted with sera diluted 1:50 in a dilution buffer (PBS + 10 % FCS + 0.1 % Tween 20 + 0.05 % KLH, w/v). KLH was added to the dilution buffer because in the initial experiments we noticed that some sera tended to react with KLH. After a 2 hr incubation at 37 °C the plates were washed and rabbit antihuman IgG or IgA peroxidase conjugates (Dakopatts, Glostrup) dissolved 1:2000 and 1:300 in the dilution buffer supplemented with 5 % normal rabbit serum were added for an additional 1 hr incubation at 37 °C. After a washing step o-phenylenediaminehydrochloride (OPD, Sigma) substrate was added for 30 min at 37 °C and the reaction was stopped with 2 N H_2SO_4. A_{492} values were read with a Multiscan photometer (Labsystems, Helsinski). The results were expressed in enzyme-immuno units (EIU) by using positive and negative reference sera (Lehtinen *et al.*, 1989). Cut-off level for positive reaction was 35 EIU for IgG and 25 EIU for IgA.

The rod-bound peptides were used in an inhibition ELISA. Briefly, sera were diluted 1:100 in PBS containing 1 % BSA, 1 % ovalbumin and 0.05 % Tween 20, and adsorbed with decreasing concentrations (3 μg/ml, 1 μg/ml, 0.3 μg/ml and 0.1 μg/ml) of free peptides for 3 hr at RT. The rods were blocked by 1 % BSA for 1 hr at 37 °C and reacted with the preadsorbed sera for 1 hr at 37 °C. The rods were washed 4 times with PBS + 0.5 % Tween 20 and 4 times with distilled water, after which rabbit antihuman IgG peroxidase conjugate (Dako) dissolved 1:1000 in the dilution buffer supplemented with 5 % normal rabbit serum was added and allowed to react for 1 hr at 37 °C. The washings were repeated after which tetramethyl benzidine (TMB, Kirkegaard and Perry, Gaithesburg) was added for 10 min at RT. The reaction was stopped with 1 N HCl. A_{450} values were measured. The rods were reused following a standardized disruption procedure (CRB manual, 1988).

Purification of adenovirus hexon antigen. Purification of adenovirus hexon antigen was performed as described by Waris and Halonen (1987). Briefly, HeLa cells were infected with adenovirus type 2. The cells were extracted after two or three days when a fully developed cytopathic effect could be found. After freon (Merck, Darmstadt) extraction and sonication the virus was pelleted by

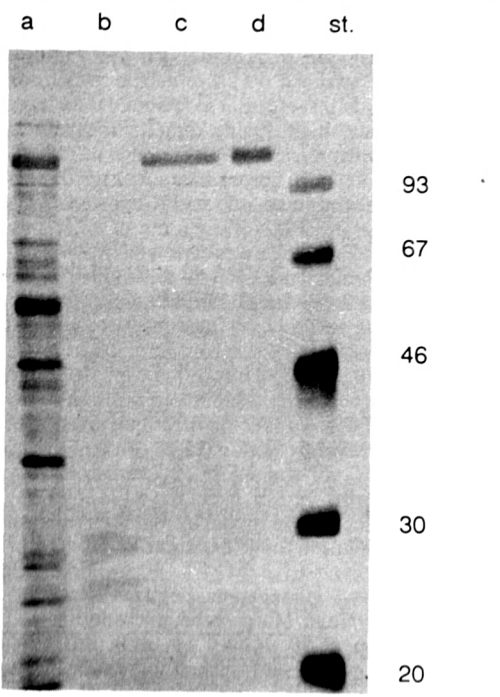

Fig. 1
Polyacrylamid gel electrophoresis of purified adenovirus hexon antigen (d)
a) freon extracted ultracentrifugation supernatant, b) low salt eluate, c) and d) high salt eluates, st) molecular weight markers (K values).

ultracentrifugation at 100 000 × g for 1 hr. Ion-exchange chromatographic purification of the hexon was done by fast protein liquid chromatography (FPLC) apparatus with Mono-Q column (Pharmacia). Polyacrylamide gel electrophoresis followed by silver staining (Lehtinen *et al.*, 1984) was used for the analysis of the purified hexon (Fig. 1).

ELISA for structural adenovirus antigen. Antibodies to a structural adenovirus antigen were measured by using the purified hexon antigen which is known to be type-common. The microtiter plates were coated with the hexon (0.1 μg/well) diluted in PBS (pH 7.2) by an overnight incubation at 4 °C. After a washing step sera diluted 1:200 in a dilution buffer (PBS + 10 % FCS + 0.05 % Tween 20) were allowed to react for 90 min at 37 °C. After a washing step rabbit antihuman IgG and IgA peroxidase conjugates diluted 1:4000 and 1:1000 were allowed to react for 1 hr at 37 °C. After an additional washing step OPD substrate was used and the reaction was stopped with 1 N HCl and A_{492} was measured. The results were expressed in EIU using positive and negative reference sera. Cut-off level for positive reaction was 20 EIU both for IgG and IgA determinations.

Statistics. Paired t-test (one-tailed) was used for the estimation of statistical significance of mean differences between case-control pairs (Lehtinen *et al.*, 1989).

Results

Characterization of the peptide ELISA

Conjugation of free peptides from their carboxyterminal and to the KLH-carrier gave the best results in the ELISA analyses. Increasing concentrations of Ad12 E1b peptide gave a clear-cut difference between positive and negative reference sera (Fig. 2). Conjugation from the aminoterminal end yielded no specific reactivity. This was the case also when the Ad12 E1b peptide was tested unconjugated. Results obtained with Ad40 E1b and A-gliadin derived peptides pointed to the same direction. Only peptides conjugated from their carboxyterminal end were reactive (Figs. 3 and 4).

Specificity of peptide-antibody reactions was further tested in an inhibition ELISA. Peptides bound from their carboxyterminal end to polyethylene rods were allowed to react with a reference positive serum which was preadsorbed with increasing concentrations of corresponding free peptides. In this kind of an inhibition assay the A values became the lower the higher the free peptide content increased (Fig. 5). The reaction was similar for both the Ad12 E1b and A-gliadin derived peptides.

On the basis of the above-mentioned results peptides conjugated from their

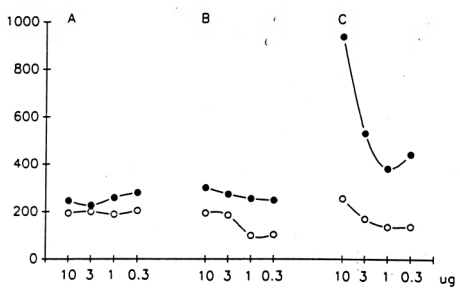

Fig. 2
Antigenic reactivity of Ad12 derived peptide (L R R G M F R P S Q C N) at different antigen dilutions
A) unconjugated peptide, B) aminoterminus conjugated peptide, C) carboxyterminus conjugated peptide. (● positive reference serum, ○ negative reference serum). Ordinate: A_{492}.

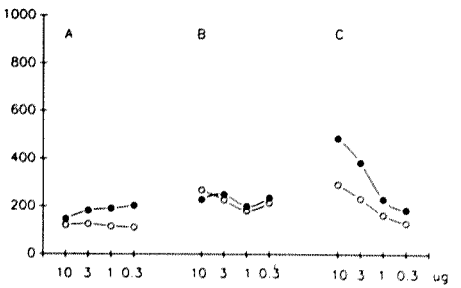

Fig. 3
Antigenic reactivity of Ad40 derived peptide (A R R G M F S P Y Q S N) at different antigen dilutions
A) unconjugated peptide, B) aminoterminus conjugated peptide, C) carboxyterminus conjugated peptide. (● positive reference serum, ○ negative reference serum). Ordinate: A_{492}.

carboxyterminal end were chosen for further analyses.

Determination of serum antibodies to non-structural and structural adenovirus antigens

Children with an acute adenovirus infection showed significantly increased antipeptide IgA antibody levels to Ad12 and Ad40 E1b compared to seronegative controls (Table 1). They also had increased IgA antibody levels to adenovirus hexon antigen. Similar trend was also seen when antipeptide IgA antibodies to A-gliadin were analyzed but the mean difference did not reach statistical significance. Ten of 16 (63 %) cases and 9 of 16 (56 %) seropositive controls had positive (>25 EIU) IgA antibody levels to the Ad E1b (results obtained with type 12 or 40 combined) derived peptide antigens, but only 2 of 16 (13 %) seronegative controls were positive. Distribution of positive antipeptide IgA antibodies to A-gliadin was almost equal among cases (44 %) and controls (25 % to 31 %).

Children with an acute adenovirus infection showed considerably increased IgG antibody levels to adenovirus hexon antigen (Table 1), but antipeptide IgG antibodies to Ad12 or Ad40 E1b failed to show major differences between cases and controls. Antipeptide IgG antibody levels to A-gliadin were somewhat higher both in the cases and in the seropositive controls when compared to seronegative controls. However, the mean differences did not reach statistical significance when adjusted to background reactivitiy obtained with KLH. Six of 16 (38 %) cases and 7 of 16 (44 %) seropositive controls had positive IgG antibody

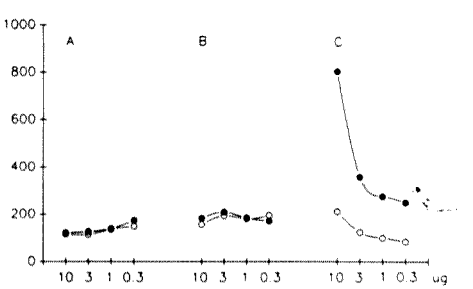

Fig. 4
Antigenic reactivity of A-gliadin derived peptide (L G Q G S F R P S Q Q N) at different antigen dilutions
A) unconjugated peptide, B) aminoterminus conjugated peptide, C) carboxyterminus conjugated peptide. (● positive reference serum, ○ negative reference serum). Ordinate: A_{492}.

Table 1. Differences in the mean adenovirus antibody levels (EIU[a]) between children with an acute adenovirus infection and their matched controls

	Cases	Controls		Differences		
	Mean (±SE)	Pos. Mean (±SE)	Neg. Mean (±SE)	Dif. 1 Mean (±SE)	Dif. 2 Mean (±SE)	
IgG						
Ad hexon	70.7 (9.2)	67.5 (4.3)	6.6 (1.4)	3.2 (9.8)	64.1 (8.4)	***
Ad12 E1b[b]	33.6 (6.1)	29.9 (4.0)	27.9 (3.4)	3.6 (8.7)	5.8 (7.6)	n.s.
Ad40 E1b[c]	33.0 (6.2)	26.9 (4.1)	22.3 (3.6)	6.1 (9.1)	10.8 (8.0)	n.s.
Gliadin[d]	45.9 (12.1)	58.3 (14.9)	24.9 (4.2)	-12.4 (21.1)	20.9 (13.9)	n.s.
IgA						
Ad hexon	20.6 (6.1)	22.9 (4.5)	6.1 (1.5)	-2.25 (7.0)	14.5 (6.5)	**
Ad12 E1b	30.3 (5.2)	24.1 (4.4)	17.8 (13.1)	6.18 (6.9)	12.4 (6.3)	*
Ad40 E1b	22.3 (3.2)	23.7 (5.8)	15.7 (1.5)	-1.5 (6.6)	6.5 (3.6)	*
Gliadin	29.1 (7.1)	24.3 (6.8)	16.8 (2.5)	6.2 (9.0)	12.3 (8.4)	n.s.

Pos. = seropositive
Neg. = seronegative
Dif. 1 = difference between cases and seropositive controls
Dif. 2 = difference between cases and seronegative controls
n.s. = not significant; * = $p < 0.05$; ** = $p < 0.02$; *** = $p < 0.0005$
[a]EIU = enzyme-immuno unit
[b]Adeno 12 E1b peptide (LRRGMFRPSQCN)
[c]Adeno 40 E1 b peptide (ARRGMFSPYQSN)
[d]Gliadin peptide (LGQGSFRPSQQM)

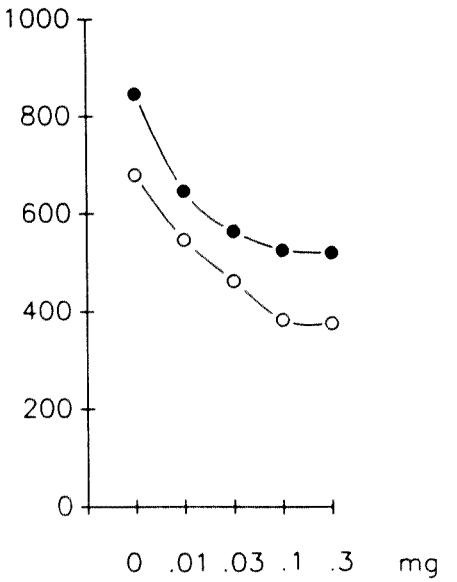

Fig. 5
Antigenic reactivity of Ad12 E1b derived peptide (L R R G M F R P S Q C N) and A-gliadin derived peptide (L G Q G S F R P S Q Q N) in an inhibition ELISA
Positive reference sera were preadsorbed with increasing concentrations of the free peptides, and thereafter reacted with polyethylene rod-bound peptides. (○ Ad12 E1b derived peptide. ● A-gliadin derived peptide). Ordinate: A_{450}.

levels (> 35 EIU) to the Ad E1b peptides (results obtained with type 12 or 40 combined), but 2 of 16 (13 %) of seronegative controls also were positive. Again there was no difference in the distribution of positive antipeptide IgG antibodies to A-gliadin in cases (44 %) and controls (31 %).

Discussion

Our results showed that adenovirus E1b protein derived synthetic peptides that correspond to the toxic fraction of A-gliadin contain B-cell epitopes. A B-cell epitope is considered to contain four amino acids at minimum (Geysen *et al.*, 1987). Our A-gliadin derived dodecapeptide contains a B-cell epitope in its carboxyterminal portion (Kagnoff *et al.*, 1987). However, according to our results B-cell epitopes appeared to be located in the aminoterminal portion of the peptides because conjugation of the peptides from their aminoterminus abolished the antigenic reactivity. Results from our inhibition ELISA analyses poin to the same direction. There was only a single amino acid difference between the aminoterminal portions of the Ad12 and Ad40 peptides, but the carboxyterminal portions of Ad12 and A-gliadin peptides also differ by one amino acid only. Exact definition of an underlying common B-cell epitope would require step-by-step scanning of the peptides.

As described by Halonen *et al.* (1979) IgA-class antibodies to the adenovirus

common hexon antigen were readily detectable soon after acute adenovirus infection. Antipeptide IgA antibodies to the Ad12 E1b were also found in the patients sera. The fact that antipeptide IgA antibodies to the Ad40 E1b showed a similar trend suggests that IgA antibodies may cross-react between E1b peptides of different adenovirus types. Combined prevalence of antipeptide IgA antibodies to adenovirus peptides was considerably higher among cases than among seronegative controls, while IgA antibodies to the A-gliadin derived peptide showed no such difference. IgA and IgM antibodies have about 10-fold lower affinity than IgG antibodies (Stanley *et al.*, 1983; Fitzgerald *et al.*, 1987; Jones *et al.*, 1987). It is possible that the relatively low affinity of IgA antibodies could facilitate cross-reactivity with adenovirus types, other than Ad12 or Ad40.

Antipeptide IgG antibody responses were more gradual than IgG antibody response to the hexon antigen. There is good evidence that antibody responses to non-structural versus structural virus antigens can differ considerably. Antibodies to Epstein-Barr virus (EBV) capsid antigen are found already at the acute phase of infectious mononucleosis, while antibodies against EBV determined nuclear antigen (EBNA) become detectable 2 to 3 months later (Henle *et al.*, 1987). Similarly in acute hepatitis serum antibodies to viral core protein can be detected early during infection but antibody response to non-structural proteins takes place 2 to 6 months after the onset of symptoms (Zuckerman *et al.*, 1987; Alter *et al.*, 1989). A plausible explanation for the lack of antipeptide IgG antibodies in a majority of adenovirus seropositive controls might be the fact that their encountered infections were caused by heterogenous adenovirus types. High affinity of IgG antibodies might not allow antigen-antibody reaction with the E1b peptides of adenovirus types 12 and 40.

In conclusion, methods for analysis of antibodies to both non-structural (E1b peptides) and structural (hexon) adenovirus antigens are now available.

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