

# Differential peptide binding motif for three juvenile arthritis associated HLA-DQ molecules

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## Abstract

### Objective

*In the oligoarticular subgroup of juvenile idiopathic arthritis, a strong association has been found with the expression of human leukocyte antigen class II molecules HLA-DQA1 \*0401-DQB1\*0402 and DQA1\*0501-DQB1\*0301, whereas DQA1\*0501-DQB1\*0201 is neutral and DQA1 \*0201-DQB1\*0201 protective. A presentation of different peptides by these DQ alleles would support their role in the disease process.*

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### Methods

*Using a synthetic nonapeptide library, a peptide binding motif was determined for the associated DQA1\*0501-DQB1\*0301 molecule and compared to the neutral and the protective DQ molecules.*

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### Results

*A differential motif for the three molecules could be deduced, suggesting that peptides preferentially binding to the associated vs. the neutral/protective DQ-molecules are mutually exclusive.*

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### Conclusion

*These results imply a role for differential peptide presentation in the pathogenesis of oligoarthritic JIA. The search for peptides initiating the disease process might be facilitated which could then lead to therapeutical interventions.*

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### Key words

HLA-DQ, juvenile idiopathic arthritis, peptide binding motif, peptide library.

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## Introduction

Juvenile idiopathic arthritis (JIA) is probably an autoimmune disease. Especially in the oligoarthritis subgroup of JIA, a strong association with alleles of at least three different regions of the human leukocyte antigen (HLA) system, namely HLA-A2 (HLA-class I), DR5, DR8, DQ4 and DQ7 (coded for in the DR/DQ region of HLA class II) and DPB1\*0201 (coded for in the DP region of HLA class II) has been described (1). The presentation of certain peptides by the susceptible HLA molecules might be an initial step in the pathogenesis of JIA with the presented peptide(s) activating (auto)reactive T cells, resulting in clonal expansion (2). Among the various HLA associations, HLA-DQ is of special interest for several reasons (3). DQ7 occurs together with DR5 and DQ4 together with DR8, i.e. a combination of DR5/DQ7 or DR8/DQ4 is inherited. Whether the primary association is with DR or DQ cannot be answered easily. Nevertheless, the alpha-chain in the DQ7 molecule (DQA1\*0501) is very similar to the alpha-chain in the DQ4 molecule (DQA1\*0401). The two different DR-molecules are therefore combined with very similar DQA-alleles. In fact, DQA1\*0401 and DQA1\*0501 show a very strong sequence homology, including a very distinct amino acid (aa)-sequence in the peptide binding groove. This sequence is found in 86% of patients compared to 36% in controls making it by far the strongest association of all HLA-alleles involved (3). Finally, a hierarchy among the DQ-molecules can be observed where DQA1\*0501-DQB1\*0301 (DQ7) shows the closest association with JIA, DQA1\*0501-DQB1\*0201 (DQ5) is neutral and DQA1\*0201-DQB1\*0201 (DQ2) protective for the disease. Such a hierarchy can not be found for the associated HLA-A2 and DPB1\*0201 alleles. A differential peptide binding motif corresponding to this hierarchical association would strongly support the hypothesis that peptides are involved in the pathogenesis of JIA and the DQ molecules are key players in initiating the disease process.

Peptide motifs in this study were deter-

mined by a recently developed acetylated nonapeptide amide library in the positional scan format (4). This library consists of 171 O/X<sub>8</sub> sublibraries (one position in the peptide is defined, O; all other positions are randomized, X) (Fig. 1), which are tested in competition with a known ligand. The binding contribution of the various aa residues can be quantified in each sequence position. The resulting peptide binding motif consists of the most favourable aa in each position. A motif based on the binding pattern for the associated DQA1\*0501-DQB1\*0301 molecule was determined and compared to the motifs of one neutral and one protective DQ-molecule.

## Material and methods

### Purification of HLA-DQ-molecules by affinity chromatography

HLA-DQA1\*0501-DQB1\*0301 (DQ7) molecules were isolated by affinity chromatography from the human cell line JO528239 (IHW 9041) (5). Briefly, cells were grown in spinner cultures in RPMI 1640 containing 2 mM L-glutamine supplemented with 10% fetal calf serum, penicillin and streptomycin. Cells were then suspended in lysis buffer and 2% Triton X 100 plus protease inhibitors. After incubation on ice for 2 h and ultracentrifugation at 35000 RPM for 30 min, the cell lysate was purified in a 4B Sepharose column and a second anti-DQ-specific mab column (6). The DQ molecules were eluted at pH 11.2. The eluate was neutralized immediately and analyzed by SDS-polyacrylamide gel electrophoresis.

### Synthesis and analysis of peptide libraries and single peptides

The synthetic peptide amides and the acetylated nonapeptide amide mixtures were prepared by multiple solid-phase peptide synthesis using Fmoc/t-butyl-oxycarbonyl-chemistry and Polystyrene A RAM amide resin (Rapp Polymere, Tübingen, Germany). The randomized sequence positions were introduced by double couplings with an equimolar mixture of 19 Fmoc-L-amino acids (cysteine was excluded) in an equimolar ratio with respect to the coupling sites of the resin. For the defined sequence

positions a 5-fold molar excess of single Fmoc-L-amino acids was used (7). Acetylation was performed by adding diisopropylethylamine (10 eq.) and acetic anhydride (10 eq.) to the resin (30 min, RT) after coupling of the ninth residue and N-terminal Fmoc-deprotection. Defined peptides were synthesized by multiple peptide synthesis as described (8). Reporter peptides were prepared by coupling biotin to the free N-terminus of the resin-bound side chain protected peptide (5 eq. each of biotin, HOBt and TBTU; 12.5 eq. of DIPEA in N-methylpyrrolidone, 2h, RT). The peptides and peptide mixtures were cleaved off the resin and the side chains were deprotected with trifluoroacetic acid/phenol/ethanedithiol/thioanisole (96:2:1:1, by vol.).

The identity of the defined peptides was confirmed by electrospray mass spectrometry (ESI-MS) and their purity was analyzed by HPLC. In order to ensure equimolarity of the amino acids in the randomized sequence positions of the peptide sublibraries, pool sequencing (9) of non-acetylated test samples and ESI-MS (10) was done.

#### Europium fluoroimmunoassay

The binding affinity of the sublibraries was tested in a Europium fluoroimmunoassay (11). Ten pmol of the HLA-DQ7 molecules and 30 pmol of the biotinylated indicator peptide for an incubation period of 24 h at pH 5.5 were found to be optimal for binding of ligands and the random library to the DQ7 molecules. After 24 h incubation at 37°C and 5% CO<sub>2</sub>, the mixture was neutralized with 10 µl 250 mM Tris-HCl (pH 8.8) and transferred to a 96-well Fluoronunc Maxisorp Plate (Nunc, Wiesbaden, Germany). This plate has been pre-coated with the monoclonal anti-DQ-antibody TU22. After washing 3 times with DELFIA washing buffer (Wallac Oy, Turku, Finland), the plate was blocked with 1% bovine serum albumin for 45 min at 37°C and washed 3 times in DELFIA washing buffer.

After 2.5 h incubation at 37°C, 2 ng of Europium-labeled streptavidin (Wallac Oy, Turku, Finland) were added and incubated for another 90 min at RT. After

washing 3 times, 100 µl DELFIA enhancement solution was added to each well and fluorescence was measured at 615 nm on a 1420 Victor Multilabel Counter (Wallac Oy, Turku, Finland).

## Results

### Peptide library and ligands

The acetylated nonapeptide amide library was designed in the positional scanning format, consisting of one totally randomized library (Ac-X<sub>9</sub>-NH<sub>2</sub>) and 171 sublibraries with one defined (O) and 8 randomized positions (X) (Fig. 1).

The biotinylated invariant chain peptide biotin-Aca-SKMRMATPLMQA-NH<sub>2</sub> (Aca: -amino caproic acid) was selected as the indicator peptide based on competitive inhibition experiments (data not shown). Subsequently, the competition of the completely randomized library X<sub>9</sub> with the biotinylated invariant chain peptide for binding to the DQ7 molecule was measured at several concentrations. A reduction in fluorescence intensity of 40%, 50% and 60% was found at a 65, 125 and 250-fold molar excesses, respectively, and these concentrations were used in all experiments with the different sublibraries (data not shown).

### DQ7 specific binding pattern of the Ac-O/X<sub>8</sub>-NH<sub>2</sub> peptide library

The competition of each sublibrary with the biotinylated invariant chain peptide was tested in the Europium fluoroimmunoassay. In a defined position, a favourable aa will lead to a stronger decrease in fluorescence than an unfavourable aa. The resulting fluorescence values were compared with the decrease of fluorescence induced by the completely randomized X<sub>9</sub> library. A relative competition value was calculated by dividing the percentage of competition of a given sublibrary with the percentage of competition of the X<sub>9</sub> library as determined in the same experiment. A value below 1 indicates an unfavourable aa residue and a value above 1 a favourable aa in a given position. The relative competition values are summarized in Table I.

There is no position where a certain aa would enhance binding very strongly,

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OXXXXXXXXX
XOXXXXXXXXX
XXOXXXXXXXXX
XXXOXXXXXX
XXXXOXXXXXX
XXXXXOXXXXX
XXXXXXOXXX
XXXXXXXOXX
XXXXXXXXXOX
XXXXXXXXXXOX

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**Fig. 1.** Acetylated nonapeptide amide library in the positional scan format.

In each sublibrary (Ac- X X X X O X X X X -NH<sub>2</sub>) eight positions are randomized (X) carrying one of 19 amino acid residues (X: = A, D, E, .....V, W, Y; cystein was omitted for synthetical reasons). One position carries a defined aa out of the 19 possible residues (O). Each of the sublibraries (19 residues x 9 positions) is composed of 19<sup>8</sup> = 1.7 x 10<sup>10</sup> different nonapeptides.

but the greatest variation of activity is seen in the core sequence of the peptide. In particular, position 4 seems to be very important for the peptide-HLA-interaction. While the negatively charged aa residues D and E strongly inhibit binding, the small residues A, G and S support binding. In contrast, the variation among different aa is lower in positions 1 to 3 with a preference for large, hydrophobic and aromatic aa (F, W, Y, I). Similarly, low variation can be observed in positions 7 to 9, where V has the highest binding affinity.

The proposed peptide binding motif for DQ 7 therefore consists of large aromatic aa in position 1, small aa in positions 4 to 6, and V in positions 7 to 9. All of these aa will not act by adding a significant amount of binding energy, but rather by meeting the difficult sterical requirements of the DQ7 molecule as further discussed below.

### Differential peptide binding pattern

To deduce a differential peptide binding pattern for the oligoarticular JIA associated DQ7-molecule versus the neutral DQ5 and the protective DQ2 molecule, we compared the present data with results that had been obtained with the same Ac-O/X<sub>8</sub>-NH<sub>2</sub> peptide library on DQ5 and DQ2 molecules in the context of coeliac disease (4). For DQ2, the DQB1\*0202 haplotype was

**Table I.** Relative competition values for the sublibraries binding to purified DQ7.

Relative competition values are shown ranked in a descending order. Values were calculated by dividing the percentage of competition of a given sublibrary with the percentage of competition of the completely randomized X<sub>9</sub> library. The calculation is based on competition data obtained at a 65-fold molar excess of the sublibraries compared to the biotinylated invariant chain peptide.

Position 1	Position 2	Position 3	Position 4	Position 5	Position 6	Position 7	Position 8	Position 9
1.4 Y	1.5 Y	1.6 A	1.7 G	1.8 G	1.6 G	1.4 A	1.4 A	1.6 V
1.4 W	1.2 W	1.5 Y	1.4 A	1.7 A	1.6 A	1.2 V	1.3 V	1.4 A
1.4 I	1.2 F	1.5 F	1.3 S	1.6 S	1.3 S	1.1 P	1.2 S	1.2 I
1.4 F	1.1 V	1.4 G	1.2 Y	1.1 T	1.2 V	1.0 G	1.2 P	1.2 F
1.3 V	1.1 I	1.3 M	1.1 N	1.0 V	1.1 I	0.9 M	1.2 M	1.1 W
1.3 G	1.0 A	1.3 I	1.1 H	1.0 M	1.0 P	0.9 I	1.2 I	1.1 P
1.2 H	0.9 M	1.2 W	1.1 F	0.9 Y	1.0 M	0.8 T	1.0 T	1.1 M
1.2 D	0.8 S	1.2 N	1.0 W	0.9 P	0.9 T	0.8 S	1.0 L	1.1 L
1.1 T	0.8 P	1.1 V	1.0 I	0.8 R	0.8 L	0.8 L	0.9 Y	1.0 Y
1.1 Q	0.8 H	1.1 H	0.9 V	0.8 L	0.8 H	0.7 R	0.9 W	1.0 S
1.1 N	0.8 G	1.0 S	0.9 L	0.8 H	0.7 R	0.7 P	0.9 G	1.0 N
1.1 A	0.7 L	1.0 L	0.8 T	0.7 N	0.7 P	0.7 F	0.9 F	0.9 P
1.0 P	0.7 E	0.7 R	0.8 R	0.7 K	0.7 F	0.6 W	0.8 R	0.8 R
1.0 M	0.5 T	0.7 P	0.8 P	0.7 I	0.6 Y	0.6 N	0.8 H	0.8 G
1.0 E	0.5 R	0.7 P	0.7 P	0.6 W	0.6 N	0.6 H	0.8 E	0.8 E
1.0 S	0.4 P	0.6 T	0.7 M	0.6 P	0.5 W	0.5 Y	0.7 P	0.8 D
1.0 R	0.4 K	0.6 K	0.7 K	0.6 F	0.5 K	0.5 K	0.6 K	0.7 T
0.8 L	0.3 N	0.6 E	- 0.1 E	0.4 E	0.4 E	0.3 E	0.4 N	0.7 H
0.8 K	0.3 D	0.3 D	- 0.3 D	0.2 D	0.2 D	0.2 D	0.4 D	0.6 K

**Table II.** Peptide binding motifs for three DQ-alleles as determined using the Ac-O/X<sub>8</sub>-NH<sub>2</sub> peptide library.

Data for DQ2 and DQ5 are taken from Terreaux *et al.* (4). In each position the most favourable and unfavourable amino acid residues for peptide-DQ-binding are shown. The mutually exclusive residues between DQ2/DQ5 and DQ7 in positions 4, 5 and 6 are given in bold.

HLA DQ	Position 1	Position 2	Position 3	Position 4	Position 5	Position 6	Position 7	Position 8	Position 9
	Favourable								
0501/0301	Y, W, I, F	F, W, Y	A, Y, F	G, A, S	G, A, S	G, A	A, V	A, V	V, A
0501/0201	F, W			D, E	Y, D	E, P	D, E		D, M, F, W
0201/0202	A, K			D, E		T,D,E,P	A, D, E,I	G	W, L, M,I
	Unfavourable								
0501/0301	D, N	N, D	D	D, E	D, E	D, E	D, E	N, D	K
0501/0201					W			M	
0201/0202			P,R	P, W, K		K, F, L, A			Q, Y, V,

taken, which only differs by one amino acid in position 135 (D to G) from DQB1\*0201. This residue is located outside the peptide binding site and should not influence peptide binding specificity. The range of relative competition values was generally greater in DQ2 and DQ5 than in DQ7. In positions 4 to 6, the negatively charged aa residues D and E, which inhibited binding to DQ7, conferred strong binding to DQ5 and DQ2. In contrast, the small residues A, G and S, favourable for binding in positions 4 to 6 to DQ7, did not significantly increase binding to DQ5 and inhibited binding to DQ2 in position 6.

In position 1, large aromatic side chains (e.g. F, W) increase binding to both DQ7 and DQ5 but not to DQ2. In position 9, the favourable residues for DQ5 and DQ2 are different from those for DQ7 but they do not inhibit binding to DQ7. In summary, the differentiation is clearest for the central core where preferred amino acids are mutually exclusive between DQ7 and DQ5/DQ2. A direct comparison between favourable and unfavourable aa for the three DQ-molecules is given in Table II.

**Discussion**

The aim of this study was to investigate whether the hierarchy of HLA-DQ

associations with JIA reflects fundamental differences in peptides presented by these molecules. Therefore, peptide motifs for three different HLA-DQ-molecules were compared, which have been deduced from allele-specific activity patterns determined with a synthetic Ac-O/X<sub>8</sub>-NH<sub>2</sub> nonapeptide library. As far as peptide interactions with HLA-class II molecules are concerned, much more data exist for HLA-DR than for HLA-DQ. The X-ray structural analysis of complexes consisting of HLA-DR molecules and the peptides presented by them shows that a core region of 9 amino acid residues interacts with the peptide binding

groove and positions 1, 4, 6 and 9 act as anchor positions (12). In contrast, our results for the DQ7-molecule show that the preferred small amino acids A and G, as well as the unfavourable residues D and E, are identical for the three central positions 4, 5 and 6 (Table I). The affinity to DQ7 depends very much on P4 with some influence of P5 and 6. Peptides with favourable aa in P 3 or P 7/8 might achieve binding without interaction with e.g. P1.

The three allele-specific aa patterns in this study are in accordance with the structural conditions of the respective DQ molecules found in computer modelling studies (13). In contrast to the DR molecules, where the specific binding requirements vary solely with the beta-chain as they all have the same alpha chain, DQ molecules are formed by different alpha and beta-chains. DQ7 and DQ5 consist of the same alpha chain but differ in their beta-chain – and as both chains contribute to the peptide binding groove, some similarities in preferred aminoacids but also some differences were to be expected. DQ2 on the other hand has the same beta-chain as DQ5 but differs in its alpha chain and completely differs from DQ7 in both chains. Our results correspond to these structural variations. In position 1, DQ5 and DQ7 prefer large, aromatic and hydrophobic amino acids, whereas the slightly different residues alanine and lysine mediate binding to DQ2. A clear differentiation can be observed in the core binding region, especially for P4-P7 where DQ7 shows a strong preference for the small aa A, G and S. In the DQ7 molecule these pockets are lined by large residues providing space only for small side chains. In contrast, the preferred negatively charged acidic aa D and E, which find basic and positively charged counterparts in the central region of DQ2 and DQ5, inhibit binding to DQ7. In P9, DQ7 will again prefer aa other than DQ2 and DQ5.

Peptide motifs for the DQ7 molecule have also been determined with other techniques. Single aa substitution studies suggested that the specificity of peptide interaction with DQ molecules

is determined by steric constraints rather than by the strong affinity of single aa residues. A significant inhibition of binding by aa D and E in positions 3-6 was found, in accordance with our data. There was no real increase in binding in any position by any aa (14). It has to be noted though, that the aa substitutions in this study have been done on a peptide with specific aa in each position (including the very favourable aa A) which by themselves may influence binding, whereas in our study all positions except the one with a specific aa were completely random. In another study, a DQ7-binding motif was determined by the alignment of 14 peptides that had been eluted from isolated DQ7 molecules (15). A wider array of aa for each position was found, probably reflecting the lack of determination of relative binding affinity by this method.

Our data add evidence for a role of distinct peptide presentation by disease-associated or protective DQ-molecules. At least two scenarios are conceivable regarding how the differential binding of peptides might influence the pathogenesis of JIA. First, putative arthritogenic peptides initiating a T-cell response ultimately leading to JIA will have a sequence that is able to bind to HLA-DQ7. DQ2 with its completely different binding requirements will not be able to bind such peptides and therefore be protective. Alternatively, during thymocyte development, DQ2 might bind an arthritogenic peptide resulting in the deletion of naive, potentially autoreactive T-cell clones. In a DQ7 environment, these clones would not be deleted, thus provoking autoimmunity. These results do not exclude an additional role for other peptides presented by other associated HLA-alleles, e.g. HLA-DR5. It is also possible that peptides derived from these alleles themselves might be presented by HLA-DQ-molecules and thus initiate the disease process (3).

Our observed binding motifs might help to identify naturally occurring proteins containing an arthritogenic peptide. The clinical implications of defining such peptides are speculative, but

different approaches such as vaccination or oral feeding to induce tolerance are conceivable.

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