

Use of the phage display technique for detection of epitopes recognized by polyclonal rabbit gliadin antibodies

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Received 27 April 1998; revised version received 13 July 1998

Abstract A random phage heptapeptide library was screened with rabbit antibodies against wheat flour proteins comprising gliadins and a small amount of low molecular weight glutenins (gli/glu). Gli/glu antibodies isolated from the sera selected different consensus sequences (CS). All CS contained tri- to pentapeptide stretches homologous to gli/glu sequences (proposed epitopes). In α - and γ -type gliadins, these sequences are clustered in the N-terminal region recently suspected to be toxic for humans with celiac disease. Peptides with CS were synthesized and checked for reactivity. Only immune and no control rabbit sera reacted with synthetic peptides. One of eight human sera containing gliadin antibodies was reactive as well (4/8 peptides) but control sera were negative. Thus the phage display technique is useful for epitope screening of polyclonal antibodies even in the case of a group of homologous but diverse antigens.

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Key words: Phage display; Peptide library; Polyclonal antibody; Gliadin; Epitope; Synthetic peptide

1. Introduction

Phage display libraries have been shown to be useful tools to investigate the interaction of ligand with defined molecules of interest. Until now, phage display libraries have mainly been used to investigate model systems applying *monoclonal* antibodies with the epitopes to be selected known in advance [1–3] or at least with antigens with known amino acid sequences [4]. The amino acid sequences selected (phagotopes) do not necessarily resemble that of the natural antigenic determinant but may mimic a discontinuous epitope (mimotopes). It would be helpful to apply the technique for elucidation of antigenic epitopes of antibodies occurring in human diseases. However, such investigations are complicated because of the *polyclonal* nature of these antibodies and because of unknown antigen. Nevertheless, recently selection of phagotopes has been described mimicking linear and structural epitopes recognized by serum-derived antibodies of rabbit [5,6] and man [7–10].

These results encouraged us to apply random peptide phage libraries to study antigenic epitopes of antibodies raised against gliadins. Gliadins are a heterogeneous group of closely related wheat storage proteins [11]. Therefore, a large number of different epitopes recognized by the antibodies may be ex-

pected. In patients with celiac disease, gliadins induce alterations of the small intestinal mucosa which may lead to malabsorption, and trigger the synthesis of antibodies against the wheat proteins and of autoantibodies. In the present report the epitope specificity was investigated of gliadin antibodies obtained by immunization of rabbits as a model for human antibodies. The aim of the study was to obtain information about the immunogenicity of the different domains of gliadins.

The results demonstrate that a number of phagotopes can be selected that are homologous to primary structures of gliadins, very probably representing antigenic epitopes, most of which are concentrated in the N-terminal region of gliadins.

2. Materials and methods

2.1. Gliadin and gliadin antibodies

A preparation of gliadins was obtained from wheat cv. Kanzler [12]. According to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [13] and immunoblot [14] the resulting product (gli/glu) contained ethanol extractable gliadins (α -, γ -, ω -type) known to be accompanied by minor amounts of gliadin-related low molecular weight (LMW) glutenins [11]. Rabbits were immunized with gli/glu [15]. Immunoglobulin G was isolated from rabbit sera by affinity chromatography on protein A. Antibodies against gli/glu (gliadin antibodies) were affinity purified using gli/glu coupled to Affigel-10 (Bio-Rad, Munich, Germany). Starting from 10 ml rabbit serum, 90 mg total IgG and 1.5 mg gliadin antibodies were obtained. Purity and specificity of isolated gliadin antibodies were checked by SDS-PAGE and immunoblot.

2.2. Phage display and sequencing

The Ph.D. Phage Display Peptide Library Kit (New England Biolabs, Schwalbach, Germany, version 1.5) was used as described [4]. After three rounds of selection, 36, 34, 67, or 65 clones (experiments 3, 9, 17, 18, respectively) were isolated and amplified. The DNA was isolated and cycle-sequenced using primers according to the instructions of the manufacturers.

2.3. Enzyme immunoassay of gliadin antibodies

Human gliadin antibodies of the IgA and IgG class were determined by enzyme immunoassay in microwells coated with gliadin as described previously [15]. The results are expressed as percentage of reactivity of a control serum provided by Labmaster (Turku, Finland). For assay of rabbit gliadin antibodies, microwells were incubated with rabbit serum (diluted 1:100 to 1:25000) and binding of immunoglobulins was demonstrated by pig anti-rabbit immunoglobulins conjugated with peroxidase (1:2000, DAKO, code P217). Serum dilution at half maximal binding is indicated as titer.

2.4. Determination of endomysium antibodies (EmA)

IgA class EmA were determined on monkey esophagus sections (Virimmun, Frankfurt, Germany) [16].

2.5. Synthesis of cellulose-bound peptides and antibody binding studies

Peptides covalently bound to a cellulose membrane via their C-

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Abbreviations: CS, consensus sequence(s); gli/glu, gliadin(s)/low molecular weight (LMW) glutenin(s)

tively). 84% of all entries containing the sequence QQFPF were prolamins.

Peptides with CS were synthesized and tested for binding to rabbit sera (Table 2). None of the immune sera recognized all of the peptides. Sera which were used for selection of phage bound to at least one of the selected peptides. One peptide

was recognized by all sera applied for biopanning experiments, two peptides were recognized by none. Testing of further sera positive for gliadin antibodies revealed binding of two sera to two peptides. Rabbit control sera reacted with none of the peptides.

To investigate if sequences recognized by rabbit antibodies

Table 1
Consensus sequences (CS) selected in biopanning experiments and postulated epitopes in gli/glu

	Experiment	<i>n</i>	Amino acid sequence							Mean frequency ^a		
										α	γ	LMW
3	CS	30	G	P	Y	S	L	L	L	0.33	0.00	0.00
	<i>I</i>		97	100	100	93	60	100	70			
	<i>S</i>		97	100	100	95	63	100	97			
	Epitope			P	Y	S						
	<i>I</i> *			100	100	93						
	<i>S</i> *			100	100	95						
9	CS 1	10	W	M	E	Q	P	F	P	0.67	4.16	0.57
	<i>I</i>		20	30	90	50	100	100	100			
	<i>S</i>		20	60	100	80	100	100	100			
	Epitope				Q	Q	P	F	P			
	<i>I</i> *				10	50	100	100	100			
	<i>S</i> *				100	80	100	100	100			
	CS 2	16	L	Q	Q	F	P	E	L	0.00	1.22	0.00
	<i>I</i>		56	75	100	100	100	100	69			
	<i>S</i>		69	75	100	100	100	100	75			
	Epitope			Q	Q	F	P	Q				
	<i>I</i> *			75	100	100	100	0				
	<i>S</i> *			75	100	100	100	100				
	CS 1	47	N	T	Q	Q	P	W	Q	0.81	0.00	0.00
	<i>I</i>		28	100	100	79	100	40	26			
	<i>S</i>		47	100	100	79	100	74	38			
	Epitope			S	Q	Q	P	Y				
	<i>I</i> *			0	100	79	100	15				
	<i>S</i> *			100	100	79	100	91				
	CS 2	11	T	Q	E	W	L	P	Q	1.27	0.17	1.42
	<i>I</i>		45	91	100	27	100	100	100			
	<i>S</i>		45	100	100	27	100	100	100			
	Epitope						L	P	Q			
	<i>I</i> *						100	100	100			
	<i>S</i> *						100	100	100			
	CS 3	7	Q	I	G	Q	Q	V	P	0.00	0.00	1.00
	<i>I</i>		100	71	100	100	57	43	47			
	<i>S</i>		100	100	100	100	86	43	57			
	Epitope		Q	L	G	Q	Q					
	<i>I</i> *		100	29	100	100	57					
	<i>S</i> *		100	100	100	100	86					
18	CS 1	34	Y	W	P	F	P	E	Q	0.95	0.11	0.43
	<i>I</i>		9	12	21	100	100	100	91			
	<i>S</i>		21	18	21	100	100	100	91			
	Epitope						P	E	Q			
	<i>I</i> *						100	100	91			
	<i>S</i> *						100	100	91			
	Epitope	20				F	P	Q	Q	0.00	2.44	0.57
	<i>I</i> *					100	100	0	91			
	<i>S</i> *					100	100	100	91			
	CS 2		A	H	M	N	P	S	P			
	<i>I</i>		32	100	100	47	37	21	21			
	<i>S</i>		37	100	100	74	37	58	21			
	Epitope				M	N	P			0.00	0.33	0.00
	<i>I</i> *				100	47	37					
	<i>S</i> *				100	74	37					

I: Percentage of the most common amino acid at a specified position in the CS. If there were two amino acids with the same frequency, the amino acid that had a higher number of similar amino acids at the respective position was further considered. *S*: Percentage of amino acids similar or identical to the most common amino acid (positive according to the BLOSUM62 substitution matrix). *I**: Percentage of amino acids in the consensus phagotopotype identical to the corresponding amino acid in the gli/glu sequences. *S**: Percentage of amino acids at a specified position of the phagotopotype similar to gli/glu. Peptide motifs in CS matching gli/glu sequences are printed in bold. *n*: Number of clones selected with CS.

^aMean frequency of epitope motifs per molecule of α -type and γ -type gliadins and of LMW glutenins. CS 1 from experiment 18 comprised eight amino acids because the consensus occurred in shifted positions within the heptapeptide.

Table 2

Reactivity of rabbit immune sera against eight different synthetic peptides with sequences according to the consensus sequences from the phage display experiments

Serum	Use in biopanning experiment no.	GPYSLLL	WMEQFPF	LQQFPPEL	NTQQPWQ	TQEWLPQ	QIGQQVP	YWPFPQQ	AHMNPSP
R60a	3	+ ^a	+	—	+	—	+	+	—
R46	9	—	+ ^a	+ ^a	—	—	—	+	—
R60b	17	+	+	—	+ ^a	— ^a	— ^a	+	—
R61	18	—	+	+	—	—	—	+ ^a	— ^a
R22	—	—	—	—	+	—	—	—	—
R47	—	—	—	+	+	—	—	—	—
R63	—	—	—	—	—	—	—	—	—
R69	—	—	—	+	—	—	—	—	—

Gliadin antibodies from the upper four sera were used for biopanning experiments.

^aSequence selected from corresponding antibodies during biopanning. Eight rabbit control sera were tested as well for reactivity with the peptides with negative results.

Table 3

Reactivity of human sera against eight different synthetic peptides corresponding to the consensus sequences from the phage display experiments

Serum	IgA gliadin antibodies	IgG gliadin antibodies	IgA EmA	GPYSLLL	WMEQFPF	LQQFPPEL	NTQQPWQ	TQEWLPQ	QIGQQVP	YWPFPQQ	AHMNPSP
H356	174	49	+	—	—	—	—	—	—	—	—
H542	135	68	+	—	—	—	—	—	—	—	—
H496	79	0	+	—	—	+	—	+	+	—	+
H1267	172	47	+	—	—	—	—	—	—	—	—
H180	232	47	+	—	—	—	—	—	—	—	—
H1370	40	165	—	—	—	—	—	—	—	—	—

Five human control sera were tested as well for reactivity with the peptides with negative results.

are epitopes for human gliadin antibodies as well, the reactivity of human sera positive for gliadin antibodies was compared with human control sera (Table 3). Only one of the gliadin antibody positive sera bound to four of the peptides. Interestingly, AHMNPSP, which was not reactive with rabbit sera, was recognized by human sera, too. Human control sera did not bind.

4. Discussion

The presence of linear epitopes in gli/glu was expected because all rabbit sera recognized proteins in ethanolic flour extracts after reduction and treatment with SDS. In keeping with that, several short contiguous stretches of 3–5 amino acids were selected that were homologous with gli/glu sequences. The degree of homology between the selected phagotopes and antigenic molecules was comparable with recent observations [6,8,9].

The significance of the selected CS as gli/glu sequences can be recognized from their frequency in gli/glu molecules. Further, for the homologous tetra- and pentapeptide motifs most of the entries in the total data base were connected with gliadins and with related prolamins from related cereal species like rye, barley, and oats. Taken together this strongly implies that selected phagotopes mimic gli/glu sequences.

Selected linear regions in CS matched well homologous regions in gli/glu sequences. However, the tripeptide homology of GPYSLLL, TQEWLPQ, and AHMNPSP with gli/glu may be too small to account for binding. In these peptides, several amino acids at positions outside the region of homology to gli/glu were selected. Thus, amino acids may contribute to the epitope more distant from the linear array but brought into

the close vicinity by the room structure of the antigen. Alternatively, tripeptide homology may exist purely by chance with the single amino acids as constituents of a mimotope.

However, a contribution of discontinuous epitopes to the antigenic repertoire of gliadin antibodies cannot be ruled out. To discover epitopes comprising a large number of discontinuous antibody binding points, libraries with longer peptide inserts may be better suited [21].

Several stretches of CS are homologous to more than one region in gliadin molecules. This is due to the repetitive structure of N-terminal domain Ib of gli/glu [10]. Thus, FPQQ and QQFPF are contained in γ -type gliadins on average three and four times, respectively. Therefore, the question arises if corresponding antibodies recognize only one or all of the homologous regions. Furthermore, QQFPF is found in α -type gliadins and LMW glutenins, too. Specificity for only one region would mean that amino acids in the neighborhood are important for recognition as well. Reactivity with more than one region of homology would mean a repetitive epitope structure and imply a high effectivity of binding of antibodies. Screening of binding sites on proenkephalin revealed more than one epitope region recognized by monoclonal antibody due to the repetitive structure of the protein [21].

Experiments with synthetic peptides demonstrate that the selected linear sequences represent structures recognized solely by sera obtained after immunization of rabbits with gliadin but not by control sera. This confirms the specificity of the selected peptide sequences as gli/glu epitopes. The sera already used in phage display experiments recognized a larger number of peptides in the luminescence assay than four further randomly chosen sera. This finding could be due the fact that the eight peptides were preselected by biopanning. This suggests

the existence of further antigenic epitopes recognized by gliadin antibodies in the latter sera. Thus LQQFPEL and NTQQPWQ may represent more often recognized epitope specificities whereas other sequences seem to be restricted to individual sera. Further, dominant clones may have overgrown less frequent ones during selection which may explain differences in the epitope repertoire indicated in Tables 1 and 2.

Experiments with human antisera revealed that only one of them contained antibodies reacting with the selected peptides. Nevertheless, this serum recognized four of the peptides at the same time, including LQQFPEL which was also bound by a large number of rabbit antisera, and AHMNPSP which was reactive with none of the rabbit sera in the luminescence assay. The different epitope specificity of human and rabbit antisera may be explained by different ways of immunization, i.e. gastrointestinal vs. intracutaneous, which may include different processing and presentation of antigen.

The distribution of postulated linear epitopes over gli/glu sequences shows clustering in the N-terminal part of α - and γ -type gliadins (Fig. 1). α - and γ -type gliadins contain three and four disulfide bridges, respectively, leading to the formation of loops and of a compact structure in the C-terminal part of the molecules [22] which may be less accessible to B-cell response than the N-terminal region. Thus, at least for antibodies recognizing linear epitopes or epitopes containing a linear component, the N-terminal region may possess a high antigenicity. Interestingly, short sequence stretches in the N-terminal region of α -type gliadins are suspected to be responsible for toxicity of gliadins in celiac patients [23]. Further investigations are currently being performed to examine the epitope specificity of human gliadin antibodies.

Acknowledgements: This work was supported by a grant of the Bundesministerium für Bildung, Forschung und Technologie (BMB+F), Interdisciplinary Centre for Clinical Research at the University of Leipzig (IZKF Leipzig) (01KS9504, Project A5). The skilful technical assistance of Mrs. P. Wels, E. Hentzschel, and B. Thiele is acknowledged.

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