# Specificity of Rabbit Antibodies Elicited by Related Synthetic Peptides

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Three 17-residue peptides, presenting from 65% to 70% sequence homology, and one endecapeptide, with no apparent homology with the first three, were chemically synthesized and investigated in their ability to elicit rabbit antipeptide antibodies.

The complex crossreactivities of the antisera were investigated by testing the binding of the antibodies to the intact peptides, to their enzymatic fragments, and by the use of specific immunoadsorbents.

Antipeptide antibodies may or may not crossreact with related "parent" peptides, this depending upon number, distribution, and localization of amino acid differences in low or high antigenicity regions of the immunogen.

Related peptides may elicit antibodies that crossreact almost completely, and therefore not specific for one or the other "parent" peptide. Those antibodies may therefore be of little use for the selective recognition of closely related structures.

## Introduction

Efforts to understand the nature of protein antigenicity have induced many investigators to assay chemically synthetised peptides to raise antibodies with predetermined specificity, and to test their ability to recognize the intact protein from which the peptide was derived [1−3].

Although a synthetic fragment with a minimal length of seven residues might be able to elicit an immune response [4], the need to maximize the possibility of producing a suitable immune serum, and to achieve an operationally-measurable binding affinity, has convinced most investigators in utilizing as immunogens synthetic peptides with 12 or more residues [5, 6].

The complexity of the immune response to a relatively long synthetic fragment composed by 36 amino acid residues, corresponding to a surface domain of a viral coat protein, has been recently well documented by the study of the specificity of the panel of monoclonal antibodies obtained [7].

However, the technique itself utilized for the selection of the antibody-forming cells may not give the true distribution and dominance of the antigenic determinants seen during the immune response. On the contrary, the study of the immune sera obtained by rabbit immunization might give a more real representation of the nature and antigenicity of different epitopes in the fragment.

Rabbit immunization with short peptides might result in the preferential production of antibodies directed against a single "strong" antigenic determinant: this fortunate event would approach the oparating condition of monoclonal antibodies. More frequently, however, multiple antigenic sites elicit heterogeneous populations of antibodies that crossreact at different extents with any antigen that exhibits a certain sequence homology with the immunogen. Those antisera may be therefore of little use for the selective recognition of closely related structures. In the present investigation, four synthetic peptides, selected from the amino acid sequence of human hystocompatibility antigens HLA−DQ1, −DQ2,−DQ3 and SB, and presenting different degrees of sequence homology, were tested in their ability to elicit specific antipeptide antibodies. The complex pattern of crossreactivity of the four antisera against the peptides and against human lymphoblastoid cell lines with known phenotype (Chersi *et al*, submitted), led us to study in details the binding

of those antisera to small-size fragments obtained from the immunogens by enzymatic digestion, and to investigate the specificity of different antibody sub-populations obtained by the use of a panel of suitable immunoadsorbents. With the obvious limitations of the method (some antigenic determinants might be cleaved by the enzymatic digestion: antibodies might, or might not, recognize part of the original site), this study gives an insight into the complexity of the immune response to a short peptide, and explains some of the reasons for low or high crossreactivity.

## Materials and Methods

### Synthesis of peptides

Three of the four peptides used in the present investigation were synthesized by using a solid-phase method [8] and a Beckman Synthesizer, model 990 B.

A single peptide (D) was prepared by manual synthesis in solution. The benzyloxycarbonyl group was used for $N^\alpha$-protection, and tertiary butyl ester for carboxyl groups. The stepwise assembly of the endecapeptide chain was carried out by using the pentafluorophenyl, the N-hydroxysuccinimide and the p-nitrophenyl esters of the protected amino acids. Every intermediate was isolated and purified.

At the end, the protecting groups were removed in two steps, first by trifluoroacetic acid and then by hydrogenolysis. The composition of each peptide was assayed by amino acid analysis after acid hydrolysis.

### Preparation of peptide fragments

According to their amino acid composition and sequence, the four peptides were digested with one or more of the following proteolytic enzymes:
1) Trypsin (3 h at 37 °C, enzyme-to-substrate ratio 2:100 in 0.1 M ammonium bicarbonate pH 8.0)
2) Chymotrypsin (6 h at 37 °C, enzyme-to-substrate ratio 3:100 in 0.1 M ammonium bicarbonate pH 8.0)
3) Staphylococcus Aureous protease (12 h at 37 °C, enzyme-to-substrate ratio 3:100, in 0.05 M ammonium bicarbonate pH 7.8.

Digestion products were separated by column chromatography on LKB AcA 202 gel filtration resin in 0.05 M ammonia. The eluate was continuously monitored at 206 nm. Composition and amount of each isolated fragment was assessed by amino acid analysis after acid hydrolysis (6 N HCl, 108 °C, 24 h) on a JEOL Analyzer.

When necessary, a further purification of the fragments was achieved by preparative paper chromatography in butanol-acetic acid-water (3:1:1).

### Rabbit immunization

Peptides A, B and C were coupled to KLH with the aid of MBS [1, 9]. Peptide D was coupled to the carrier protein by the use of glutaraldehyde.

Those peptide-protein complexes were then used to immunize New Zealand white rabbits: details of the immunization are similar to those described in previous papers [10].

### Preparation of the immunoadsorbents

Peptides A, B and C were bound to AH-Sepharose 4B through their C-terminal cysteine by the use of MBS. Briefly, 4 ml of AH-Sepharose, a gel with a six carbon atom "spacer" and an amino group available for coupling, were reacted in 0.05 M phosphate buffer pH 7.1 with 2 mg MBS (5 mg/ml in DMF), under gentle stirring. After 3 h, the gel was washed by repeated centrifugations, and reacted with 2 mg peptide in 2 ml buffer, for 12 h.

The immunoadsorbent, equilibrated in PBS, was then routinely used in column chromatography to purify specific antipeptide antibodies from the immune sera, according to the method previously described [11].

A different approach was employed for all enzymatic fragments of the four peptides, since the majority of them lacked a free −SH group. All those fragments were therefore linked to an activated derivative of Sepharose 4B through their amino groups.

Briefly, each fragment (0.6−0.8 mg) in 2 ml of 0.1 M NaHCO$_3$− 0.5 M NaCl buffer, was added under stirring to 1 ml of previously activated N-hydroxysuccinimide derivative of Sepharose 4B. The suspension was stirred gently for 12 h at 4 °C. The immunoadsorbent was then recovered by centrifugation. The same technique was employed also for peptide D, which lacked a cysteine group.

### Binding test

Binding of the antipeptide antibodies to the antigens was routinely assayed by the Enzyme Linked Immuno Sorbant Assay. Wells of microtiter plates

were coated with 2 µg of synthetic peptide or 1 µg of small-size fragment. The test was carried on as originally described by Church *et al.* [1].

## Results

After three immunizations with KLH-peptide complexes, the antisera were tested in ELISA against the free peptides. All antisera were able to recognize their own antigen, but most of them cross-reacted also with other fragments. In particular, extensive crossreaction was observed between antisera 203, 207 and peptides B and C: in contrast, peptide A was scarsely recognized by these antisera. At the same time, antiserum 202 reacted very poorly with B and C (Fig. 1).
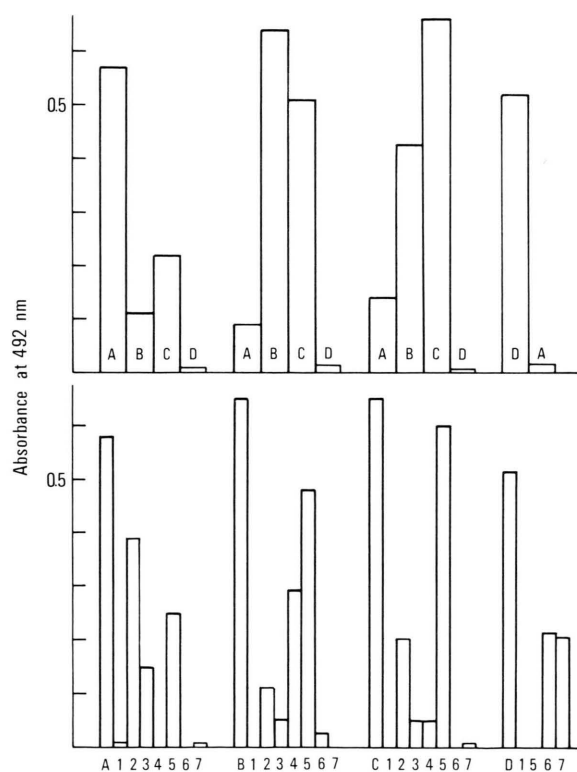


Fig. 1. Upper part: Binding of antisera 202, 203, 207 and 208 to the four peptides, as evaluated in ELISA. Immune sera were diluted 1:100, then used in the amount of 50 µl. Wells of Microtiter Plates were coated with 2 µg of peptide. The assay was carried on as described by Church [1]. Lower part: Binding of antisera to peptide fragments obtained by enzymatic digestion. Wells were coated with 1 µg of fragment. 1: A/1. 2: A/2. 3: A/3. 4: B/4. 5: C/5. 6: D/6. 7: D/7. The binding to the immunizing peptide is also reported for comparison.

This result could not be initially interpreted, since the degree of homology among the three peptides was about the same (70% between A and B, 65% between A and C, 70% between B and C) (Table I). No crossreaction occurred between the first three antisera, and peptide D, which in fact had no sequence homology with any of the first three peptides.

In restricted regions of the peptides, the degree of homology may be as high as 100% (as for instance fragment 1−7 S Q K E V L E, common to A and B), or as low as 40% (as for instance fragment 7−13 of peptide A, compared to the same fragment in B). Some antigenic sites might therefore be recognized by more than one antiserum, and some not. To analyze this possibility, we tested the binding of the four antisera to small-size fragments obtained from the synthetic peptides by enzymatic digestion. The data are reported in Fig. 1, and can be summarized as follows:

− none of the antibodies binds to hexapeptide S Q K E V L
− the fragment B/4 (E V L E G A R), although showing over 70% homology with the corresponding fragment in A, is not recognized by antiserum 202 (anti-A).
− All three antisera 202, 203 and 207 bind, to different extents, to the heptapeptide A/2 (E R T R A E L).

Table I. Amino acid sequence of the peptides (immunogens) and of fragments obtained by enzymatic cleavage.

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | S | Q | K | E | V | L | E | R | T | R | A | E | L | D | T | V | C |
| B | S | Q | K | E | V | L | E | G | A | R | A | S | V | D | R | V | C |
| C | S | Q | K | D | I | L | E | R | K | R | A | A | V | D | R | V | C |
| D | A | F | S | F | E | A | Q | G | G | L | A | | | | | | |
| A/1 | S | Q | K | E | V | L | | | | | | | | | | | |
| A/2 | | | | | | | E | R | T | R | A | E | L | | | | |
| A/3 | | | | | | | | | | | A | E | L | D | T | V | C |
| − | | | | | | | | | | | | | | D | T | V | C |
| − | S | Q | K | | | | | | | | | | | | | | |
| B/4 | | | | E | V | L | E | G | A | R | | | | | | | |
| − | S | Q | K | D | I | L | E | | | | | | | | | | |
| C/5 | | | | | | | | | R | K | R | A | A | V | D | R | V | C |
| D/6 | A | F | S | F | E | | | | | | | | | | | | |
| D/7 | | | | | | | A | Q | G | G | L | A | | | | | |

Peptides A, B and C correspond to the amino acid sequence of histocompatibility antigens HLA-DQ3, HLA-DQ1 and HLA-DQ2 (pos. 63−79 of the β chain). Peptide D to HLA-SB (pos. 51−61 of the α chain). Fragments with less than 5 amino acid residues as D T V C (from peptide A) or S Q K (from peptide B) were not investigated. Fragment S Q K D I L E (from peptide C) was not used for binding assays because obtained in low yield.

Sera: 202: anti-A, 203: anti-B, 207: anti-C, 208: anti-D.

– Antiserum 203 reacts with fragment C/5. This fragment is also partly recognized by antiserum 202, although the degree of homology between peptides A and C, between pos. 8–17, is only 60%.

– The binding of antiserum 202 to A/2 approaches 65% of the binding to the entire peptide A, that of antiserum 207 to C/5 almost 95% of that to peptide C.

– Antiserum 208 binds to both fragments D/6 (A F S F E) and D/7 (A Q G G L A). The antibody specificities seem to be quite equally distributed between the two regions. These data are confirmed by inhibition assays: both fragments D/6 and D/7 inhibit the binding of antiserum 208 to peptide D by a similar extent, approaching, at high concentrations, 40% inhibition (data not shown).

As a control of the binding data, affinity-purified antibody 202 (anti-A) and 208 (anti-D) were treated with a panel of immunoadsorbents prepared by linking different enzymatic fragments of the peptides to Sepharose. After reaction and centrifugation, the residual binding ability of the immunodepleted antibodies was tested again in ELISA against the antigen peptides. The data (Fig. 2) can be summarized as follows:

– Immunodepletion of antibody 202 by Seph-A/1, Seph-A/2 and Seph-A/3 resulted in a loss of binding activity of approx. 5%, 60% and 25%. No change was detected after immunodepletion with Seph-B/4, Seph-D/6 and Seph-D/7.

– Immunodepletion of antibody 208 by Seph-D/6 and Seph-D/7 resulted in a loss of binding activity of 30% and 40% respectively. No change in binding ability was observed by immunodepletion with Seph-A/2, Seph-A/3, Seph-B/4.

In the last experiment, aliquots of affinity-purified antibody 203 (anti-B), each containing a total protein amount of 2.4 mg, were loaded on columns containing either Seph-A, or Seph-C, or Seph-D immunoadsorbents. This procedure might separate crossreacting antibodies from not crossreacting antibodies [12].

On Seph-A columns, the first fraction, eluted with PBS (65% of the total protein amount loaded) reacted with B and did not bind to peptide A, as expected. On Seph-C columns, only 7% of the protein loaded was eluted by PBS, and this fraction lacked not only anti-C activity, but almost also anti-B activity. In few words, almost all antibodies elicited by peptide B, crossreacted with antigenic sites of peptide C, and were therefore retained.

On Seph-D, 95% of the protein loaded was eluted immediately with PBS, as expected.

## Discussion

The usefulness of an antipeptide antibody may be dependent on its ability to recognize selectively a sequence of a limited number of amino acids. Crossreactions with related or unrelated substances should be ideally absent.
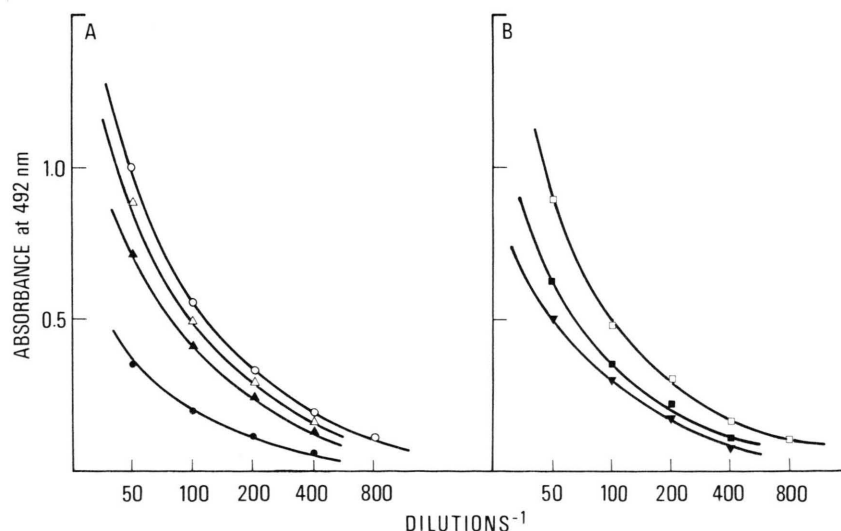


Fig. 2. A) Binding of antibody 202 to peptide A, before (○) and after immunodepletion by Seph-A/1 (△), Seph-A/2 (●) and Seph-A/3 (▲), as evaluated in ELISA. B) Binding of antibody 208 to peptide D, before (□) and after immunodepletion by Seph-D/6 (■) and Seph-D/7 (▼).

When using as immunogens synthetic peptides with close similarity in amino acid sequence, the elicited antipeptide antibodies may exhibit quite close specificities. It might happen, however, that related peptides generate antibodies that do not crossreact. As an example, peptides A and B (5 amino acid differences, 70% homology), elicit antibodies that scarcely crossreact, while antisera 203 and 207, raised against two peptides with comparable amino acid substitutions, show very close specificities.

Evidently, for the generation of crossreacting antibodies, the number of amino acid differences between two immunogens is not as determining as their position and distribution: occurrence of common amino acid sequences in certain regions of the peptides may have little effect. Peptides A and B have in common the first seven amino terminal residues, while differences are located between pos. 8 and 15. Since the two antisera 202 and 203 poorly crossreact with the two peptides, it might be suggested that the amino terminal region did not elicit antibodies. This is confirmed by the fact that neither antiserum 202 nor 203 bind to the fragment A/1. Again, fragment B/4 (E V L E G A R) is not recognized by antiserum 202, although peptides A and B share, in that region, 5 of the 7 amino acids (72% homology).

Most commonly, however, closely related peptides elicit antibodies with similar specificities, and even regions with low homologies may be recognized by crossreacting antibodies. This event is well documented by the low but still measurable binding of antiserum 203 (anti-B) to fragment A/2 (pos. 7−13 of peptide A), which shows only 42% homology with the corresponding fragment in B. Evidently, some antibody populations elicited by peptide B are able to recognize somehow the three amino acid residues E − − R A − − common to both fragments. Again, antiserum 202 (anti-A) binds to C/5, recognizing therefore some of the common residues R − R A − − D − V C. Binding of antipeptide antibodies to not-related antigens has been previously reported, as the result of fortuitous similarities in short amino acid sequences [11].

Finally, the impossibility in obtaining from antiserum 203 (anti-B), by the use of Sepharose-peptide C immunoadsorbents, a population of antipeptide antibodies able to recognize specifically some characteristic antigenic determinants of peptide B, indicates that the probability of producing truly specific reagents, able to discriminate closely related structures, as the products of allelic genes, might be low. It may be difficult to predict whether two structurally-related peptides will elicit antibodies with similar specificities, or not. Probably the selection as immunogens of shorter peptides with the lowest degree of homology with related antigens will increase the specificity of many antipeptide antibodies.

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