Brief Definitive Report

PEPTIDES AS ANTIGENS
Importance of Orientation

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The use of peptide antibodies in biomedical research has the major theoretical and practical advantage that one can direct antibody production to predetermined amino acid sequences, thereby obtaining site-specific immune reagents (1). The main requirement for producing peptide antibodies is knowledge of the amino acid sequence of the protein or part thereof. Such site-directed antibodies, induced by immunization with synthetic peptides, have proved useful for (*a*) determining the protein(s) encoded by the open reading frame of a nucleic acid sequence (2); (*b*) establishing the orientation of proteins in the plasma membrane (3, 4); (*c*) distinguishing between closely related proteins that differ by only a single or a few amino acids (5); (*d*) designing immunodiagnostic reagents (6); (*e*) determining the proteolytic cleavage sites of proteins (7); and (*f*) analyzing the molecular basis of virally and microbially induced autoimmunity (8, 9).

Not all attempts to raise protein-reactive peptide antibodies have been successful (10, 11), however, and many failures occur despite attention to selecting amino acid sequences of appropriate size, charge, and helix formation. While investigating the sharing of amino acid sequences between virus proteins and a defined region of the α chain of the human acetylcholine receptor (HuAChR), we noted (T. Dyrberg, P. Schwimmbeck, and M. B. A. Oldstone, manuscript in preparation) that the orientation of the immunizing peptide was important for the generation of antibodies. Here we describe the immune response to the same peptide when coupled through a Gly-Gly-Cys linker at either the *N*- or *C*-terminal end, to a carrier protein. From these observations it is clear that orientation of the immunizing peptide is one of the essential considerations for raising antipeptide antibodies.

Materials and Methods

Peptide Synthesis. Peptides (Table I) were synthesized by using an automated peptide synthesizer (430A; Applied Biosystems, Foster City, CA), and their purity was confirmed by HPLC analysis. Peptides used for immunization were synthesized with either an *N*- or *C*-terminal linker consisting of a cysteine residue and two intervening glycine residues as spacers.

Immunization and Immunochemical Analysis. Peptides were coupled to a carrier protein (KLH) via the terminal cysteine residue using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide

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TABLE I
Synthetic Peptides Used as Immunogens and Antigens

Peptide name	Residues
N-HuAChR 160–167	C G G P E S D Q P D L
C-HuAChR 160–167	P E S D Q P D L G G C
HuAChR 160–167	P E S D Q P D L
HuAChR 160–168	P E S D Q P D L S
HuAChR 157–170	A I N P E S D Q P D L S N F

The peptides represent sequences from the α chain of the HuAChR. Rabbits were immunized with either *N*-HuAChR 160–167 or *C*-HuAChR 160–167, which were synthesized with two glycine residues as spacers and an *N*- or *C*-terminal cysteine residue through which the peptide was coupled to a carrier protein.

ester (12), and they were used for immunizing New Zealand White rabbits within 3 wk after coupling. After obtaining preimmune sera, groups of three were immunized as follows: on day 0, each rabbit was injected intradermally (i.d.) and subcutaneously (s.c.) with 200 μg peptide–carrier protein in 1 ml of CFA; day 14, injected i.d./s.c. with the same dose of coupled peptide in IFA; and on day 21, injected i.p. with 200 μg coupled peptide in 1 ml of an alum suspension. Rabbits were bled 14 d after the last immunization, serum was obtained, divided into aliquots, and stored at −20°C until use.

The presence and specificity of peptide antibodies were tested by ELISA. Briefly, a 96-well microtiter plate was coated with 0.5 μg/well of peptide not coupled to carrier protein. Unbound reactive sites were blocked by incubation with 0.2% Tween 20, 10% FCS, and 0.5 mM thimerosal in PBS (ELISA buffer). The plates were incubated with various rabbit sera diluted in ELISA buffer, and unbound material was removed by washing five times with 0.2% Tween 20 in PBS. Peroxidase-labeled affinity-purified goat anti–rabbit IgG was added, and finally the plates were washed five times and substrate was added. Negative controls included preimmune sera and incubations without first and/or second antibodies. In competition assays, selected amounts of the competing peptides were added along with the first antiserum. Finally, peptide antisera binding of AChR from *Torpedo california* (kindly donated by Dr. J. Lindstrom, Salk Institute, La Jolla, CA) was tested as cited (4).

Results

Antisera from all rabbits immunized with either *N*-terminal HuAChR residues 160–167 or *C*-HuAChR residues 160–167 (defined in Table I) reacted strongly with the immunizing peptide, however, analysis of the *N*-coupled peptide antiserum's binding to the *C*-terminal peptide and vice versa demonstrated a negligible crossreactivity (Fig. 1, *A* and *B*). These results were confirmed by competition assays in which either the immunizing or the homologous free peptide was used to inhibit binding of antibodies to bound peptide. For both groups of peptide antisera, it was found that at a concentration where the immunizing peptide completely inhibited binding, the homologous peptide with the Gly-Gly-Cys linker in the opposite orientation did not decrease the binding compared to buffer alone (Fig. 2).

To further characterize the binding properties of the peptide antisera, we tested their reactivity with various shorter or longer peptides (Table I). When the eight-amino-acid core peptide, HuAChR residues 160–167, was used as antigen, antisera to *N*-HuAChR 160–167 bound significantly, whereas antisera to *C*-HuAChR 160–167 showed no binding (Fig. 1*C*). Competition analysis showed that HuAChR 160–167 inhibited the binding of anti-*N*-HuAChR 160–167 to *N*-HuAChR 160–167 as effectively as *N*-HuAChR 160–167 itself, whereas HuAChR 160–167 did not inhibit the binding of anti-*C*-HuAChR 160–167 to *C*-HuAChR 160–167 (Fig. 2). The addition of a *C*-terminal serine residue to

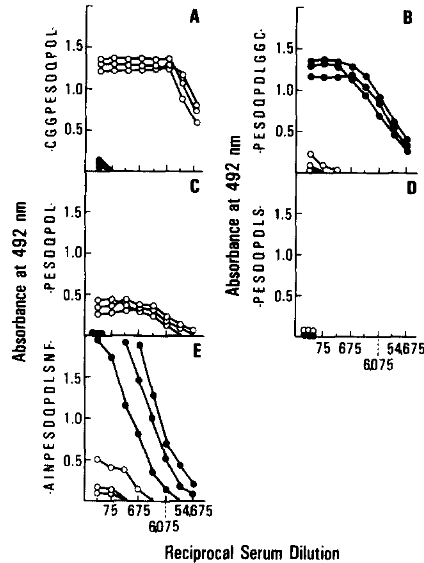

FIGURE 1. Binding, in an ELISA, of peptide antisera to homologous peptides derived from the same region of the receptor (listed in Table I). Rabbits ($n = 3$) were immunized with synthetic peptides representing the α-chain of the HuAChR positions 160–167, coupled through either an *N*- or *C*-terminal linker to carrier protein. *A*, *N*-HuAChR 160–167; *B*, *C*-HuAChR 160–167; *C*, HuAChR 160–167; *D*, HuAChR 160–168; *E*, HuAChR 157–170; (○) antiserum to *N*-HuAChR 160–167; (●) antiserum to *C*-HuAChR 160–167.

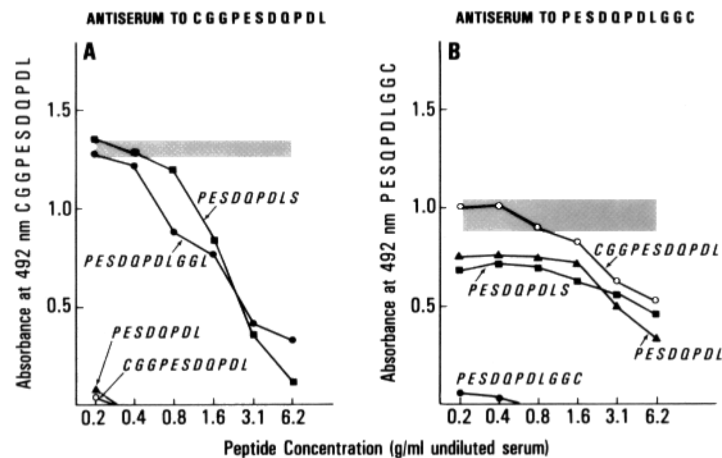

FIGURE 2. Competition assay of peptide antiserum binding to the immunizing peptide in ELISA (see Materials and Methods). The shaded area represents binding with buffer alone. Peptide concentration is expressed as grams per milliliter undiluted serum calculated according to volume and dilution of first antiserum. For peptide sequences see Table I. *A*, inhibition of *N*-HuAChR 160–167 antiserum binding to *N*-HuAChR 160–167. Final serum dilution 1:40,000. *B*, Inhibition of *C*-HuAChR 160–167 antiserum binding to *C*-HuAChR 160–167. Final serum dilution 1:10,000.

HuAChR 160–167 (Table I) resulted in a complete lack of binding of both anti-*N*-HuAChR 160–167 and anti-*C*-HuAChR 160–167 to this peptide, HuAChR 160–168 (Fig. 1*D*), and in competition analysis, HuAChR 160–168 did not inhibit the binding of either peptide antisera to their homologous peptides (Fig.

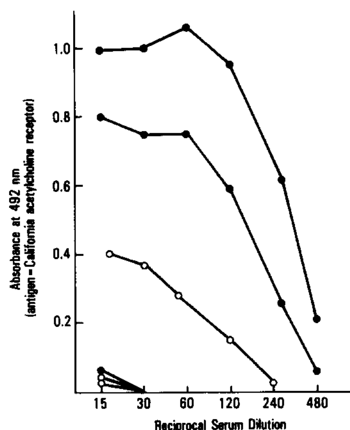

FIGURE 3. Peptide antisera reactivity to native protein tested by using *T. californica* AChR as antigen in an ELISA. Rabbits were immunized with synthetic peptides representing the α chain of the HuAChR, positions 160–167, either coupled through an *N*- or *C*-terminal linker to carrier protein. The binding induced by preimmune serum is subtracted. (○) antiserum to *N*-HuAChR 160–167; (●) antiserum to *C*-HuAChR 160–167.

2). Analysis of the binding to a 14-amino-acid peptide representing the α-chain of HuAChR 157–170, including the 8-amino-acid core sequence (Table I), showed that antisera to *N*-HuAChR 160–167 bound negligibly to HuAChR 157–170, in contrast to antisera to *C*-HuAChR 160–167, which bound strongly (Fig. 1*E*).

With *T. californica* AChR used as the antigen, antiserum to *N*-HuAChR 160–167 did not bind (two of three rabbits) or bound weakly (one of three) (Fig. 3). In contrast, the two rabbit antisera to *C*-HuAChR 160–167 that reacted most strongly with HuAChR 157–170 also showed marked binding to the *T. californica* AChR (Fig. 3).

Discussion

We have shown that the orientation of synthetic peptides to carrier proteins can influence their properties as antigens. In our studies, despite close homology between the two immunizing peptides, the resulting peptide antibodies show very little crossreactivity (Fig. 1) owing to the amino or carboxy orientation for peptide coupling. The pattern of binding to shorter or longer homologous peptides suggests that immunization with the *N*-terminal-coupled peptide induces antibodies reactive with the free *C*-terminal amino acid residue, and that their binding depends on the identity and charge of the amino acid. In contrast, immunization with the *C*-terminal-coupled peptide induces antibodies that react with an epitope near the *C*-terminus of the peptide, and this binding is probably conformation-dependent.

The sequence of the peptides used as immunogens (Table I) originates from a region of the α chain of the HuAChR thought to be a major immunogenic site. Immunization of rabbits with these peptides coupled to the carrier by either an *N*- or *C*-terminal linker resulted in antisera that reacted with the immunizing peptides at comparable titers. However, only antisera directed against the *C*-terminal-coupled peptide reacted significantly with a peptide representing a 14-amino-acid sequence of the HuAChR (Fig. 1*E*) and with the AChR from *T.*

californica (Fig. 3). Despite the binding of the anti-*C*-terminal antibodies to both the extended HuAChR peptide and to the *T. californica* AChR in an ELISA, none of the peptide antibodies bound ^{125}I-bungarotoxin–labeled receptor in solution (data not shown). Thus, this region of the receptor may not be a major immunogenic site.

Several events are important in determining whether a peptide antibody will bind to the native protein from which the peptide sequence is derived. First, the length of the immunizing peptide is critical. To raise antibodies to a peptide, a minimum length of six amino acids is required, and peptides of >10 amino acids generally induce antibodies that may bind to the native protein (10). Equally important is the part of the protein from which the sequence is derived. Antibodies raised against an accessible epitope have a better chance of binding to the native protein than antibodies to an inaccessible site, however, the process of antibody binding itself may render inaccessible sites accessible. Third, as we show here, the site where a peptide is coupled to a carrier protein is of importance. Here we illustrate that the direction in which peptides are coupled to carrier proteins affects the binding characteristics of the peptide antibodies, not only with respect to the initiating peptides but also, more importantly, to the native protein.

Our analysis of the binding pattern of the peptide antibodies suggests that the immunogenic sites on the immunizing peptide may be restricted to single amino acids. This supports the finding that individual amino acids may be epitopes, either directly or through their influence on neighboring amino acids (5). Thus, the immune response against short peptides results in antibodies with specificity at the level of the individual amino acids. The implications of this conclusion are twofold. First, finer characterization of the protein structure is possible more than ever before, e.g., in deducing cleavage sites on precursor molecules. Second, the governing factor(s) in immunological tolerance to a given antigen can be studied at the molecular level by analyzing binding of autoantibodies to synthetic peptides representing sequences from their natural autoantigens.

Summary

Factors known to be important in producing protein-reactive peptide antibodies include the accessibility of the region from which the peptide sequence is derived, the hydrophilic-phobic character of the sequence, and the length of the peptide. The data presented here indicate that the orientation of the peptide coupled to a carrier protein also influences the binding pattern of peptide antibodies. An octapeptide, representing a sequence from the α chain of the human acetylcholine receptor, was coupled either through an *N*- or *C*-terminal cysteine-glycine-glycine linker to a carrier protein and used to immunize rabbits. The resulting antisera reacted at comparable titers to the uncoupled immunizing peptides, but did not crossreact with the identical but opposite-linked peptide. Characterization of the binding to other homologous peptides showed that immunization with the *N*-terminal-linked peptide induced antibodies reactive specifically with the *C*-terminal amino acid(s). Immunization with the *C*-linked peptide resulted in antibodies reactive with a site of the peptide near the *C*-terminus.

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