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Journal of Autoimmunity 24 (2005) 47–54

journal of autoimmunity

www.elsevier.com/locate/issn/08968411

A peptide of human muscarinic acetylcholine receptor 3 is antigenic in primary Sjögren's syndrome

Ilona Marczinovits[a,*], László Kovács[b], Andrea György[c], Gábor K. Tóth[d], László Dorgai[c], János Molnár[a], Gyula Pokorny[b]

[a]*Department of Physiology, University of Szeged, Szeged, Hungary*
[b]*Department of Rheumatology, University of Szeged, Szeged, Hungary*
[c]*Institute for Biotechnology, Bay Zoltán Foundation for Applied Research, Szeged, Hungary*
[d]*Department of Medical Chemistry, University of Szeged, Szeged, Hungary*

Received 28 July 2004; revised 9 November 2004; accepted 15 November 2004

Abstract

To evaluate the antigenicity of a peptide representing a part of the second extracellular loop of the human muscarinic acetylcholine receptor 3 (m3AChR) with autoimmune sera from primary Sjögren's syndrome (pSS), enzyme-linked immunosorbent assays (ELISAs) were developed. On the basis of the computer-predicted data, a 16-mer synthetic peptide KRTVPPGECFIQFLSE ($KRSE^{213-228}$) was produced by solid-phase peptide synthesis. cDNA coding for the KRSE peptide was chemically synthetized and utilized to express the recombinant glutathione S-transferase (GST)–KRSE fusion protein. The immunoreactivities of the two antigens were tested in ELISAs with the sera of 40 pSS patients and 40 healthy controls. The specificity of the reaction was confirmed by inhibition assays and immunoblottings.

The pSS sera resulted in significantly higher mean optical densities than those of the healthy controls (KRSE: 0.4149 vs 0.1494, $p < 0.0001$; GST–KRSE 0.4765 vs 0.1764, $p < 0.0001$). The immunological recognition with the recombinant fusion antigen was significantly better than that for the free peptide ($p = 0.0068$). The sensitivities of the assays were 77.5% (KRSE) and 97% (GST–KRSE). The results of the concentration-dependent inhibition assays by the two systems of peptide presentation indicated that the KRSE sequence is specific for pSS sera. This is the first demonstration of the antigenicity of a novel peptide fragment of the human m3AChR in pSS. The analysed peptide could be of diagnostic relevance.

Keywords: Peptide; Antigenic; ELISA; Primary Sjögren's syndrome

1. Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease. A large number of autoantibodies have been reported to be frequently present in pSS. In particular, antibodies to the autoantigens SSA/Ro and SSB/La, found most commonly in pSS, are used in the diagnosis of the disease, although these antigens have also been linked to other autoimmune diseases. The recent finding of muscarinic acetylcholine receptor subtype 3 (m3AChR)-specific autoantibodies in a majority of the patients is an important advance towards an understanding of the pathogenesis of pSS, as concerns not only the impaired glandular function, but also the associated features of an autonomic dysfunction in some patients [1].

The detection of antibodies against the human m3AChR in the sera of patients with pSS has led to

* Corresponding author. Department of Anatomy, Laboratory of Molecular Neurobiology, Faculty of Medicine, University of Szeged, H-6724 Szeged, Kossuth L. sgt. 40, Hungary. Tel.: +36 62 545 665; fax: +36 62 545 707.
E-mail address: ilona@comser.szote.u-szeged.hu (I. Marczinovits).

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doi:10.1016/j.jaut.2004.11.005

controversial results in the peptide ELISA system [2,3]. The diagnostic significance of these antibodies has therefore remained uncertain.

Synthetic peptides have increasingly been used as antigens in ELISAs. However, the results are often inconsistent. Reliable immobilization of the peptides on a solid support poses difficulties, and peptide–plastic interactions often alter the correct exposure of the epitopes in a manner that is difficult to predict. Covalent coupling of the peptides to a carrier protein is therefore expected to improve the sensitivity of the assays by providing an appropriate orientation and density of the epitope [4]. Peptides are often coupled to glutathione S-transferase (GST) for expression in bacteria as fusion proteins which are easy to overexpress and purify.

Our previous results involving the use of recombinant technology for the production of peptides fused to GST for the diagnosis of bullous pemphigoid support the advantages of the fusion strategy [5–7].

With regard to the contradictory results of the detection of m3AChR antibodies by peptide ELISA and our encouraging experience with the recombinant fusion peptide, we decided to test whether a novel amino acid sequence, KRSE$^{213-228}$, representing a part of the second extracellular loop of the human m3AChR, is sufficiently antigenic to bind autoantibodies from the sera of pSS patients. For this purpose, we presented the peptide in two forms, the synthetic peptide and its recombinant fusion variant, as antigens.

2. Patients and methods

2.1. Patients

Sera were collected from 40 pSS patients (38 females, 2 males, mean age: 55 years, range: 30–82 years). They all fulfilled the American–European classification criteria for pSS [8]. Serum samples from 40 healthy blood donors (mean age: 49 years, range: 23–62 years) served as controls. All patients gave their informed consent. The study was approved by the Medical Ethics Committee of the University of Szeged.

2.2. Computer analysis and peptide synthesis

The sequence of the human m3AChR was from the Swiss-Prot databank (Acc. No P20309) and the amino acid sequence (m3AChR$^{191-250}$) was analysed with the program "Peptide Companion" Version 1.231 (Coshisoft/PeptiSearch).

The peptide sequence of KRTVPPGECFIQFLSE (KRSE, m3AChR$^{213-228}$) was synthetized by a solid-phase technique, utilizing tBoc chemistry [9]. The peptide chains were elongated on a *p*-methylhydrylamine resin (0.48 mmol/g), using an ABI 430A automatic machine. The crude peptide was purified by reverse-phase HPLC and was characterized by mass spectrometry. The detailed procedure is to be found in [6].

2.3. Recombinant techniques

cDNA coding for the KRSE peptide was chemically synthetized and cloned as described by Molnár et al. [7]. The recombinant fusion product consists of GST (pGEX-6P-1, Pharmacia) coupled with the antigenic peptide at the C-terminus. A dipeptide spacer coded by the *BamH*I site used for cloning (Gly-Ser) was inserted between the GST and the monomeric peptide sequence. The GST and the recombinant fusion proteins were expressed in *Escherichia coli* as described earlier [10]. The fusion protein was purified over a glutathione-Sepharose affinity matrix (Pharmacia) from the soluble fraction of *E. coli* cell lysate and dialysed against phosphate-buffered saline (PBS, pH 7.4) containing 10 mM dithiotreitol (DTT) before being used for ELISA.

2.4. Purification of anti-KRSE monospecific antibody from pSS sera

The GST Orientation kit (Pierce) was used, which leads to the covalent attachment of the GST–KRSE fusion protein to glutathione immobilized on a matrix (cross-linked 4% beaded agarose). For the covalent cross-linking of the affinity-purified GST–KRSE and the purification of the peptide-specific antibodies, the procedure suggested by the instructional manual was followed.

2.5. PAGE and Western blotting

Expressed and affinity-purified GST and GST–KRSE fusion proteins fractionated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were stained with Coomassie brilliant blue (Sigma) or transferred to nitrocellulose membrane (0.2 μm, Schleicher and Schuell). The membrane was blocked with SuperBlock Blocking Buffer (Pierce), and incubated either with monospecific antibodies (1:200) or with patient's serum (1:500), preincubated with 500 μg/ml GST in blocking solution. This was followed by incubation with peroxidase-labelled goat anti-human IgG (1:10 000, Sigma) and the assay was developed with 3,3′-diaminobenzidine tetrahydrochloride.

The synthetic peptide applied for the ELISA was either reduced with 50 mM DTT or its cysteine residues were oxidized in the air. Their electrophoretic mobilities were analysed by 15% SDS–Tricine PAGE [11]. Peptide was transferred from the gel to nitrocellulose membrane (0.2 μm). Transfers were carried out according to [12] with minor modifications, in 25 mM sodium phosphate