Correspondence

Letters

INSTRUCTIONS FOR LETTERS TO THE EDITOR

Editorial comment in the form of a Letter to the Editor is invited; however, it should not exceed 800 words, with a maximum of 10 references and no more than 2 figures (submitted as camera ready hard copy per Journal Guidelines) or tables and no subdivision for an Abstract, Methods, or Results. Letters should have no more than 3 authors. Full name(s) and address of the author(s) should accompany the letter as well as the telephone number, fax number, or E-mail address.

Contact. The Managing Editor, The Journal of Rheumatology, 920 Yonge Street, Suite 115, Toronto, Ontario M6J 3G7, CANADA. Tel: 416-967-5155; Fax: 416-967-7556; E-mail: jrheum@jrheum.com Financial associations or other possible conflicts of interest should always be disclosed.

Total Hip Arthroplasty in Ankylosing Spondylitis

To the Editor:

I read with interest the recent paper by Sweeney, Gupta, Gordon, and Calin[1]. They identified 309 patients who had undergone total hip arthroplasty (THA) from the National Ankylosing Spondylitis Society database of 4569 (6.7%) members. Of this total, the 237 with known addresses and complete data were sent a questionnaire mainly focusing on the hip arthroplasty. Of the 237 contactable database members, 166 replied (112 men, 54 women). These 166 patients had undergone a total of 340 hip replacements, including 64 revisions.

Can the authors please explain their claim in the title of this paper that they are reporting the outcome of THA in 340 patients with ankylosing spondylitis?

MUHAMMAD ASIM KHAN, MD. Case Western Reserve University School of Medicine, Cleveland, Ohio, USA.

REFERENCE

1. Sweeney S, Gupta R, Taylor G, Calin A. Total hip arthroplasty in ankylosing spondylitis: outcome in 340 patients. J Rheumatol 2001;28:1862-6.

Dr. Calin replies

To the Editor:

Professor Khan is of course correct to point out that at some stage a typographical error occurred and the article refers to 340 total hip replacements, rather than 340 patients. Somewhere the word "hips" was replaced by "patients." Naturally, the conclusions of the study remain intact. Specifically, the longterm outcome in total hip arthroplasty in ankylosing spondylitis is outstandingly good. I am sorry for the confusion.

ANDREI CALIN, MD, FRCP. Royal National Hospital for Rheumatic Diseases, Bath, BA1 1RL, England.

Failure to Detect Antibodies to Extracellular Loop Peptides of the Muscarinic M3 Receptor in Primary Sjögren's Syndrome

To the Editor:

Recent evidence suggests that autoantibodies directed against muscarinic M3 receptors (M3R) may contribute to sicca and autonomic symptoms in primary Sjögren's syndrome (SS)[1]. These autoantibodies have been demonstrated in the non-obese diabetic mouse[2], but their existence in patients with SS remains controversial. We have recently shown that the immunoglobulin fraction from both primary and secondary SS patients inhibits carbachol-evoked bladder smooth muscle contraction by about 50%, as well as inhibiting the effect of neuronally released acetylcholine at the M3R, consistent with autoantibodies that act as antagonists at the M3R[1]. Since the functional assay used in these studies is too laborious for routine use, simpler automated assays for anti-M3R autoantibodies are required for screening large patient populations. Because the second extracellular (ec) loop of G protein-coupled receptors (GPCR) has been reported to be an epitope for anti-receptor autoantibodies in certain autoimmune disorders such as Chagas' cardiomyopathy[3], neonatal lupus[4], and idiopathic dilated cardiomyopathy[4], we tested SS sera for reactivity against the second ec loop of the M3R. Binding of antibodies to the second ec loop of the M3R was reported in patients with primary and secondary SS[5], but these authors used a synthetic peptide corresponding to the muscarinic M4 receptor (M4R), rather than the M3R[7], due to an incorrect entry in GenPept (accession no. S10127).

Sera were obtained, after informed consent, from 33 patients with primary SS who fulfilled at least 4 of the 6 European consensus criteria (including the presence of anti-Ro/SSA and anti-La/SSB antibodies)[6]; 13 patients with rheumatoid arthritis (RA) and secondary SS; 19 patients with RA; 12 patients with systemic lupus erythematosus (SLE), and 40 healthy controls. Streptavidin-coated ELISA plates (Nunc, Roskilde, Denmark) were coated with 20 µg/ml of the following biotinylated peptides for 60 min at 37°C in 0.03 M carbonate buffer, pH 9.6: QYFVGKRTVPPGECFIQFLS ($M3R^{208-227}$) and KRTVPPGECFIQFLSEPTITFGTAI ($M3R^{213-237}$), corresponding to the second ec loop of the M3R (GenPept accession no. P20309). The peptides were synthesized by solid phase peptide synthesis using F-moc based chemistry with biotin at the NH_2-terminus, purified by reverse phase high performance liquid chromatography and verified by amino acid analysis. Molecular ion mass of the peptides was confirmed by electrospray ionization mass spectrometry (Mimotopes, Clayton, Australia). After blocking with 1% bovine serum albumin, the wells were incubated with 2-fold serial dilutions of rabbit sera starting at a dilution of 1/500, or human sera diluted 1/100 in 1% blocking buffer for 2 h at 37°C. Bound immunoglobulin was detected with an alkaline phosphatase-conjugated goat anti-human IgG (Sigma, St. Louis, MO, USA) or sheep anti-rabbit Ig (Silenus, Melbourne, Australia). A rabbit polyclonal antibody was raised against the human M3R second ec loop peptides and used as a positive control for the anti-M3R peptide ELISA.

New Zealand rabbits (n = 2) were immunized subcutaneously with 500 µg $M3R^{208-227}$ and $M3R^{213-237}$ peptides emulsified in complete Freund's adjuvant and boosted twice with peptides emulsified in incomplete Freund's adjuvant at 28 day intervals.

The rabbit polyclonal serum, but not the pre-bleed, reacted strongly

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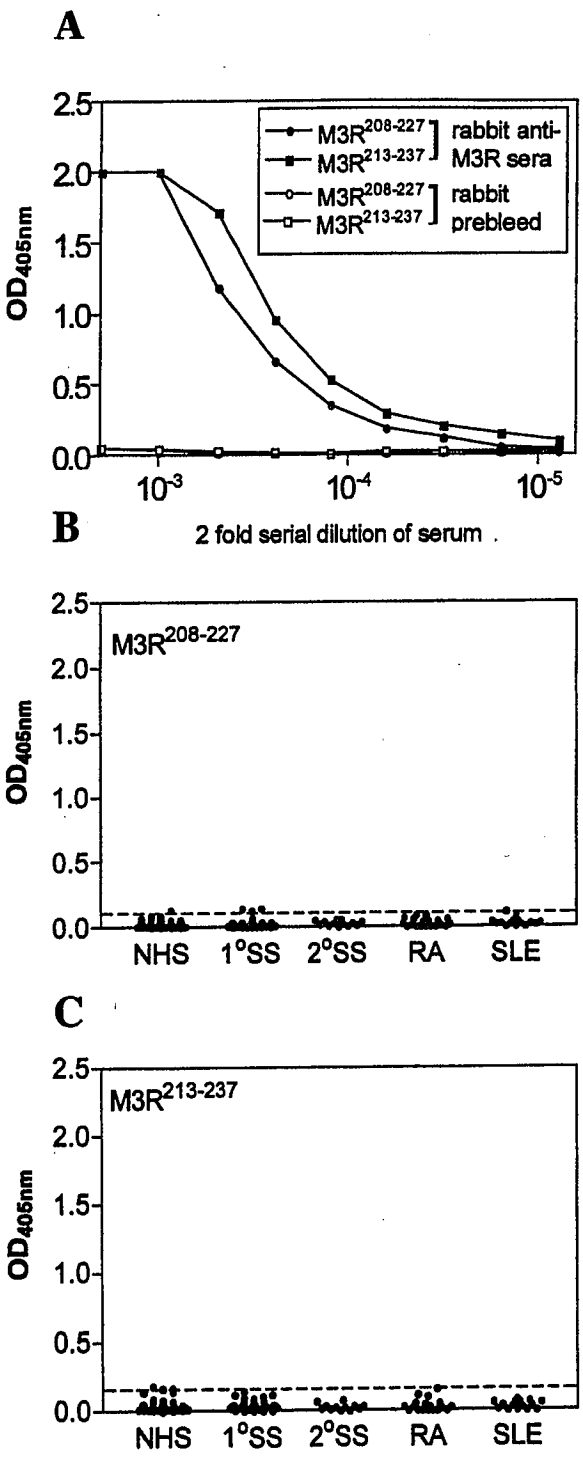

Figure 1. Reactivity of rabbit anti-M3R control serum and human sera with biotinylated second extracellular loop peptides of the M3R coated to streptavidin ELISA plates. A. Reactivity of rabbit antisera and pre-bleed sera with $M3R^{208-227}$ and $M3R^{213-237}$. B and C. Human sera from patients with primary SS (1°SS, n = 33), rheumatoid arthritis with secondary SS (2°SS, n = 13), rheumatoid arthritis (RA, n = 19), systemic lupus erythematosus (SLE, n = 12), and healthy controls (NHS, n = 40) were tested in duplicate at a 1/100 dilution and optical density (OD) read at 405 nm. The dashed horizontal lines indicate 3 standard deviations above the mean for the 40 healthy subjects.

with both M3R peptides by ELISA (Figure 1A); the reactivity was abrogated by absorption with 100 μg/ml of both M3R peptides, but not by parallel absorption with a control peptide (data not shown). Interestingly, the rabbit anti-M3R peptide polyclonal serum did not react by flow cytometry with a cell line constitutively expressing surface M3R (data not shown), suggesting that the second ec loop in the native receptor has a different conformation to the linear peptide. Optical density (OD) readings were taken for human sera (1/100) when the rabbit control serum (1/2000) reached an OD_{405nm} of 2.0 units, about 30 min after the addition of substrate. Sera from the patients with primary SS gave low OD readings on both M3R peptides that were not significantly different from the healthy controls (Figure 1B and C): $M3R^{208-227}$ (primary SS: mean ± SD: 0.029 ± 0.034; healthy controls: 0.020 ± 0.019); $M3R^{213-237}$ (primary SS: 0.044 ± 0.037; healthy controls: 0.023 ± 0.017) (Mann-Whitney U test, $p > 0.05$). No reactivity was detected in sera from patients with RA and secondary SS, RA or SLE (Figure 1B and C). To rule out the presence of low concentrations of M3R autoantibodies in patient sera, which may be missed by fixed time incubations, OD readings were also taken for human sera after 4 h, when a 1/32,000 dilution of rabbit control serum reached an OD of 2 units. Again, the OD readings of the primary SS patients were not significantly different from the healthy controls: $M3R^{208-227}$ (primary SS: mean ± SD: 0.295 ± 0.253; healthy controls: 0.298 ± 0.247); $M3R^{213-237}$ (primary SS: 0.329 ± 0.294; healthy controls: 0.354 ± 0.238) (Mann-Whitney U test, $p > 0.05$). Five patients in the primary SS group and 6 patients with RA and secondary SS had been shown previously to have autoantibodies acting as functional antagonists at the M3R in the smooth muscle bladder assay[1]. Consistent with the results obtained by ELISA, the patient sera did not bind the M3R peptides on immunoblots (data not shown). Moreover, sera from the primary SS patients did not bind by ELISA to M3R peptides synthesized as non-biotinylated monomers or 8 branched multiple antigenic peptides, indicating that the biotin moieties were not masking epitopes on the peptides (data not shown).

Since the first and third ec loops have been reported to be epitopes on other GPCR autoantigens[9], we tested the primary SS sera on peptides corresponding to the first and third ec loops of the M3R. As with the second ec loop peptides, no significant reactivity was detected against the first ec loop (primary SS: 0.055 ± 0.031; healthy controls: 0.038 ± 0.022) or third ec loop (primary SS: 0.050 ± 0.033; healthy controls: 0.045 ± 0.032) of the M3R. In addition, sera from the SS patients did not bind to a peptide corresponding to the second ec loop of the M4R (KRTVPDNQCFIQFLSNPAVTFGTAI) (GenPept accession no. CAA33336), even after long duration protocols with substrate as described above (data not shown). The M4R peptide shows 76% homology with the second ec loop of the M3R.

Autoantibodies against the M3R have been reported in patients with SS based on radioligand binding studies on rat salivary glands[10] and mouse bladder smooth muscle functional assays[1], leading to attempts to define the epitopes using linear peptides. The lack of reactivity with the M3R and M4R linear peptides in the present study is in accordance with earlier studies on the thyrotropin receptor, the GPCR autoantigen in Graves disease, in which reactivity is directed against conformational epitopes on the native receptor[11,12]. A disulfide bond links the first and second ec loops of the native M3R and may be critical for ligand binding and proper receptor cell surface localization as shown by mutagenesis studies[13]. We therefore speculate that putative autoantibodies against the M3R are directed against conformational epitopes, possibly consisting of the disulfide-linked first and second ec loops. Alternatively, complex conformational epitopes may be formed following dimerization of the M3R[14]. Finally, we cannot exclude the possibility that reactivity on the functional assay is not directly with the M3R but with associated proteins, resulting in inhibition of parasympathetic neurotransmission to smooth muscle.

In summary, simple peptide-based immunoassays cannot replace the more complex functional or ligand binding assays for the detection of anti-M3R antibodies. Further confirmation of human autoantibodies against the M3R will require immunoprecipitation of the native receptor, but this

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approach is currently hampered by the lack of specific receptor toxins to label the M3R.

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