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Neurotransmitter Receptors – A Unique Pathway of Neuro-Immune Interactions

László Kovács*
Faculty of Medicine, Department of Rheumatology, Albert Szent-Györgyi Health Centre, University of Szeged, Szeged, Hungary

Abstract. Circulating autoantibodies directed against receptors of various autonomic neurotransmitters, including the muscarinic and nicotinic ganglionic acetylcholine-receptor and the beta-1-adrenergic receptor have been detected in patients with various autoimmune diseases. These antibodies have been hypothesised to have a direct influence on the function of the target organ, including exocrine glandular function, smooth muscle or myocardium contractility. The potential immune-mediated function-modifying interaction between the immune and the autonomic nervous system has inspired interesting experiments. These studies have explored unique pathways whereby autoantibodies may alter the cellular and tissue homeostasis leading to organ damage. The present review summarises these results, with particular focus on anti-muscarinic receptor antibodies in Sjögren's syndrome, and highlights the difficulties in the detection of anti-neurotransmitter receptor antibodies.

INTRODUCTION

Autoantibodies that react with postsynaptic receptors of autonomic nervous system neurotransmitters have increasingly attracted attention in the past few years. These include antibodies to various muscarinic acetylcholine-receptor (mAChR) subtypes, ganglionic nicotinic receptors and beta-1-adrenergic receptors. These autoantibodies are supposed to modulate the function of the involved organs by means of interfering with the autonomic neurotransmission. Long-term effects of this interaction may lead to the deprivation of autonomic neural stimuli that may result in profound structural remodelling of the target organs. The present review focuses on the functional and immunodiagnostic properties of anti-mAChR autoantibodies in systemic autoimmune diseases, in particular in Sjögren's syndrome (SS) and will provide an outlook into further diseases affected by autonomic neurotransmitter receptor antibodies.

SS is a chronic autoimmune multisystem disease. The cornerstones of the disease are dry eyes and mouth leading to keratoconjunctivitis sicca and xerostomia, respectively, which are consequences of a dramatic decrease in the tear or saliva production, respectively. Further exocrine glands (including nasal, bronchial, vaginal glands etc.) and other tubulo-epithelial structures (i.e. biliary tree, renal canaliculi, bronchopulmonary system, thyreoid gland) are also frequently affected. The chronic autoimmune process often leads to immune-complex mediated manifestations, including vasculitis, neuritis, glomerulonephritis, and eventually may evolve into lymphoma, which is approximately 40 times more common than in the general population [1].

The diagnostic hallmark of SS is the demonstration of a focal lymphocytic infiltrate in the salivary glands. A peculiar finding in SS is that a critically low exocrine function is associated with a seemingly

*Correspondence to: László Kovács, MD, PhD, Faculty of Medicine, Department of Rheumatology, Albert Szent-Györgyi Health Centre, University of Szeged, Kálvária sgt. 57, H-6725 Szeged, Hungary. Tel./Fax: +36 62 561 332; E-mail: kovacs.laszlo@med.u-szeged.hu.

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modest glandular destruction, where glandular areas infiltrated with lymphocytes are separated with extensive areas of histologically normal salivary gland tissue [2]. This finding raises the obvious hypothesis that the loss of glandular function is not merely a direct consequence of the destruction of the secretory system, but, to a certain extent, also of a functional inhibition of fluid secretion. Inspired by the example of Chagas' disease, a chronic parasitic infection caused by Tripanosoma cruzi, in which chronic autonomic dysfunction develops mediated by antibodies directed to beta-adrenergic and cholinergic receptors [3], Bacman et al. were the first to demonstrate that immunoglobulin-G (IgG) from SS patients are able to react with the mAChR of rat parotid gland [4]. These antibodies have been found to inhibit the binding of a cholinergic agonist to mAChR in a non-competitive manner, and to increase the phosphoinositide turnover and decrease the cAMP levels. These findings were confirmed *in vivo* in the elegant experiments of Robinson et al.: various knock-out strains of the non-obese diabetic (NOD) mouse (a model of SS) were studied, and they found that the NOD-Igμ-null mice had preserved salivary flow despite the salivary gland destruction and lymphocytic infiltration identical to that seen in wild-type NOD-mice, and the salivary flow became severely diminished when IgG from SS patients was infused in these mice [5]. Moreover, the IgG fraction responsible for the elicitation of the salivary dysfunction was identified to competitively inhibit the action of a mAChR agonist. These findings suggest that antibodies that block cholinergic neurotransmission are present in the sera of SS patients, and these antibodies are necessary for the evolution of salivary gland hypofunction in an animal model of SS.

The first human experimental data on a parasympathetic dysfunction derive from our experiments using the skin microcirculation as a model of the cholinergic innervation. We examined the dilatation of cutaneous vessels in response to the intracutaneous administration of a cholinergic agonist [6] and we observed a significantly impaired microvascular reaction to cholinergic stimulation in SS patients. In fact, exactly half of the patients proved to be totally unresponsive. We speculated that an autonomic neuropathy is unlikely to be the cause of this finding, because the cholinergic agonist was administered directly into the target organ. The role of inflammatory mediators was also considered unrealistic, as the examined organ was located distinct from the involved glands. A dysfunction at the receptor or post-receptor level of signal transduction was therefore presumed, and we suggested that autoantibodies that react with the acetylcholine receptors may be responsible for the unresponsiveness to cholinergic stimuli in SS.

FUNCTIONAL STUDIES ON ANTI-mAChR ANTIBODIES IN SS

Further characterisation of the putative anti-mAChR antibodies were carried out in two major pathways: 1) functional studies including *in vivo* experiments on animal models, and functional assays on various cell lines or tissues and 2) the immunodetection and the determination of the epitope-specificity of these autoantibodies.

Further experiments on NOD mice provided quite convincing confirmation of the strong impact of anti-m3AChR antibodies on the pathogenesis of the salivary dysfunction in this model. Nguyen et al. tested the influence of the administration of various monoclonal antibodies on the saliva flow in NOD-*scid* mice [7]. Due to the *scid*-mutation, these mice retain their capability of saliva production. The infusion of monoclonal antibodies to the subtype-3 mAChR (m3AChR) – the functionally dominant one of the 5 known mAChR subtypes in the salivary and lachrymal glands – resulted in a marked reduction of saliva flow, whereas anti-La (a diagnostic marker of SS), anti-parotid secretory protein antibodies had no impact on the saliva production. Another group transferred splenocytes from m3AchR–/– mice immunised with murine m3AChR-peptides into Rag1–/– mice. Saliva production decreased and a marked infiltration of inflammatory cells (predominantly Th1 and Th17 CD4+ T-helper cells) evolved in the salivary glands. When only CD3+ T-lymphocytes were transferred, the above-mentioned sialadenitis did develop, but saliva production remained intact indicating that B-cell-derived factors are indispensable for the evolution of the full clinical picture of the disease in mice [8].

Microfluorimetry, patch-clamp and immunofluorescence confocal microscopy studies have consistently proved, that IgG from SS patients inhibit the cholinergic agonist-evoked intracellular calcium-accumulation, K+ and Cl– channel activation in both rodent and human salivary gland cells [9–11]. This action is mediated by the m3AChR. This selectivity is further supported by the finding that other pathways, such as the adenosine-receptor-mediated cellular activation, were not influenced by SS IgG (9). The

consequences of the impaired acetylcholine-induced salivary acinar cell-activation include a defective water secretion, as evidenced by the impairment of carbachol-induced aquaporin-5 translocation in human salivary gland cells pre-incubated with SS IgG [11]. The importance of this finding is highlighted by the fact that reduced aquaporin-5 concentrations at the apical membrane of the acinar cells in the lachrymal and salivary glands (due to impaired trafficking of the molecule to the cell surface) are thought to decrease tear and saliva production in SS patients [12]. Further cellular alterations in SS IgG-pretreated cells are a diminished cytoskeletal reorganisation of α-fodrin, a process that normally takes place after cholinergic stimulation. Anti-fodrin antibodies have been found to be relatively specific of SS, although, due to its low sensitivity, it has not become routinely used. Anti-m3AChR antibodies have been shown to induce further alterations in the salivary glands: m3AChR-specific IgG affinity-purified from SS patient sera stimulated the production of matrix-metalloproteinase-3 in submandibular gland cultures [13]. This effect was mediated by prostaglandin-E2 via phospholipase-A2 and cyclooxigenase-2 activation. These results raise the possibility that anti-m3AChR antibodies may participate in the structural destruction of the exocrine glands as observed in SS.

The inhibition of parasympathetic neurotransmission by SS-IgG has also been confirmed in bioassays using smooth muscle cells as targets. Using isolated bladder strips, Waterman et al. revealed that IgG from SS patients inhibited the smooth muscle contraction evoked by either parasympathetic nerve stimulation or by a cholinergic agonist [14]. Sera from patients with various autoimmune connective tissue diseases with sicca symptoms also demonstrated this inhibition, while from those without sicca symptoms did not. Control experiments suggested that this inhibitory effect was mediated through the muscarinic receptor. In a further set of examinations, SS-IgG also inhibited the neuronally mediated or agonist-evoked gastrointestinal motility in a m3AChR-dependent fashion as evidenced by the disrupted contractility of isolated muscle strips or whole-organ colonic motility in mice [15].

An important question is the response of the salivary acinar cells to chronic exposure to anti-m3AChR antibodies as it putatively happens in the human disease. Indeed, Beroukas et al. found the expression of m3AChR to be up-regulated in SS salivary glands as compared with controls [16]. In contrast, a recent publication reports on the induction of receptor-internalisation in response to long-term incubation with SS IgG in salivary gland acinar cells [17]. The process of receptor-internalisation is a well-known phenomenon during chronic agonist-exposure, and the internalised receptors later either recirculate to the surface or are degraded. In the study of Jin and colleagues, the amount of both surface and intracellular m3AchR decreased due to progressive receptor phosphorylation, clathrin-coat dependent internalisation and probably a subsequent intracellular degradation of m3AChR in response to 90 minutes' exposure to anti-m3AChR positive SS serum. Moreover, it was demonstrated in NOD-mice (in which anti-m3AChR antibodies are present) that cholinergic agonist-evoked responses are diminished as modelled by the contraction of isolated bladder strips as compared with preimmune NOD-mice or control strains [18]. This finding was consistent with receptor desensitisation due to the chronic stimulation by circulating anti-m3AChR antibodies. All these findings provide interesting mechanisms by which autoantibodies reactive with autonomic nervous system receptors could exert their action, but the long-term effects of anti-m3AChR antibodies on the function and the structure of the target organ need more clarification.

IMMUNODETECTION OF ANTI-m3AChR ANTIBODIES

As it could be seen, several results have emerged concerning the functional changes in response to IgG obtained from SS patients or relevant experimental animals, but, obviously, studies on anti-m3AChR antibodies would require the reliable isolation and identification of these antibodies. A convincing proof of their pathogenetic role would be the development of a validated immunodiagnostic method to test the prevalence, concentration, and epitope-specificity of these autoantibodies, what would ultimately lead to a useful diagnostic test in the clinical practice. However, the results of these efforts have remained quite controversial so far.

The first report suggesting the existence of antibodies to the acetylcholine-receptor utilised an isotopic receptor binding assay to examine the reaction of rat salivary gland homogenates with SS sera [4]. The first ELISA method for the detection of anti-m3AChR antibodies was reported by Bacman et al, who found anti-m3AChR antibodies in the majority of the examined primary and secondary SS patients, but not in healthy controls or rheumatoid arthritis patients [19]. Following the example of Chagas' disease or Graves

disease - in which the immunodominant epitope is localised at the second extracellular loop, which also contains the ligand-binding region – they selected a 25-mer peptide from this section of the molecule, and used it as an antigen in a peptide-based ELISA. However, a few years later, it turned out that this peptide was actually a peptide sequence of the human m4AChR [20]. Cavill and co-workers tested both the m3AChR-peptide with the correct amino-acid sequence and the erroneously chosen m4AChR-peptide and failed to demonstrate a consistent immunoreaction with either of the peptides despite several trials of laboratory methodological approaches [20]. Dawson et al. later reached the same conclusion after the negative results of multiple immunological approaches, including Western blot and spot-blot tests of m3AChR-transfected Chinese hamster ovary cell membrane fragments or a whole cell ELISA in which these cells were applied as antigen [21]. A subsequent report, however, contradicted these negative conclusions: Gao and co-workers succeeded in demonstrating the presence of anti-m3AChR antibodies in 9 of 11 SS patients by using a fairly sophisticated ELISA system in which the antigen was a Chinese hamster ovary cell line transfected with a recombinant human m3AChR protein propagated in hygromycin [41]. To complicate the issue further, another two articles from this period both reported positive results on anti-m3AChR antibodies albeit in relatively small proportions of SS patients [16/95 and 11/122, respectively] [22, 23] following the use of a peptide ELISA with the same 25-mer peptide as that with which Cavill et al. were unable to detect anti-m3AChR antibodies in SS patients. The epitope-specificity also remains elusive as the third extracellular epitope was also suggested to be an immunodominant region of the molecule [24] with surface plasmon resonance studies, and others have also found an immunoreaction between the N-terminal region, the 1st and 3rd extracellular loop and SS sera, although with variable functional consequences [25]. To circumvent the difficulties with linear synthetic peptides, cyclic peptides were also generated corresponding to various segments of the m3AChR with some degree of reactivity with SS or other autoimmune sera [26]. But after extensive examinations from Illei's group using several forms of various m3AChR-derived peptides, the authors concluded that peptide-based ELISAs are not sensitive and specific enough to detect m3AChR autoantibodies in serum from patients with SS [27].

We have also utilised multiple ELISA methods in order to test whether the putative anti-m3AChR antibodies actually react with the human antigen, and to assess the prevalence of these autoantibodies in SS and various healthy and disease control populations. In view of our previous experience with peptide ELISAs [28], we hypothesised that an epitope that is possibly conformational and not linear can better preserve its immunogenic characteristics in a system that provides a protein microenvironment for the antigenic sequence rather than using the peptide itself, as in ELISAs involving relatively short synthetic peptides as antigen. Accordingly, through the use of computer software, we predicted the immunodominant amino acid sequence of the m3AChR molecule, and on this basis, we selected a 16-mer peptide at the 2nd extracellular loop of the m3AChR as an antigen. We then constructed a recombinant fusion protein containing this peptide fused with glutathione-S-transferase (GST), and compared the performances of ELISA systems using this antigen and the same peptide chemically synthesised. The peptide ELISA posed numerous methodological difficulties and, although we detected anti-m3AChR213-228 antibodies in a high proportion of primary SS patients, we concluded that this mode of antigen presentation is rather problematic and not fully reliable [29]. However, with the ELISA in which the recombinant GST-m3AChR213-228 fusion protein was used as antigen, we were able to demonstrate specific and reproducible binding reactions with SS sera, indicating the presence of anti-m3AChR213-228 antibodies in 90% of the primary SS patients and in none of the controls [30]. Moreover, the incidence and mean levels of these antibodies were significantly lower in patients with rheumatoid arthritis, systemic lupus erythematosus or undifferentiated sicca syndrome.

Our experience during these measurements confirmed that it is a rather problematic task to develop an ELISA system for the detection of anti-m3AChR antibodies, which explains the conflicting results obtained earlier. We speculated that the probable immunogenic epitope of the antigen should be a conformational epitope, and the appropriate conformation provided by a protein microenvironment, such as a GST-fusion protein structure, is necessary for the assumption of the natural conformation required for the immunogenicity of the antigen. This may be in part a consequence of the two cysteine residues within the epitope, and our experiments suggested that oxidation or the dimerisation of the peptide may lead to crucial changes in its applicability as an antigen [29]. This may also explain the markedly lower levels of anti-m3AChR positivity in assessments with the peptide ELISA as compared

with the methods in which the epitope was "embedded" in a more natural microenvironment, as in the cases with cell-based [28] or GST-bound [29, 30] antigen presentation.

Even though the presence of circulating m3AChR-specific antibodies has not been convincingly proven, a further confirmation of their pathogenicity would be to demonstrate the presence of anti-m3AChR antibodies in the target organ, i.e. the salivary glands. As polyclonal SS IgG is expected to bind extensively to the salivary tissue at multiple localisations, the verification of the specific binding of serum IgG to the m3AChR would require ultrastructural studies in which the receptor is clearly identifiable. We therefore used electron-microscopic immunocytochemistry, and were able to demonstrate the binding of IgG in SS sera to the postsynaptic membrane of the junction of the end-plates of cholinergic nerves on human salivary gland acinar cells, and also *in vivo* bound IgG in SS salivary gland biopsy specimens at the above-mentioned localisation corresponding to the m3AChR [31]. These findings seem to support that anti-m3AChR antibodies are not only detectable in the sera of SS patients, but they are also able to react with their putative autoantigenic targets.

ANTI-m3AChR ANTIBODIES IN SYSTEMIC SCLEROSIS (SCLERODERMA)

SS does not appear to be the only disease where anti-m3AChR antibodies might be relevant. Another systemic autoimmune illness, systemic sclerosis (SSc), characterised by excessive diffuse skin and soft-tissue fibrosis, and a dramatic microcirculatory disturbance leading to ischaemic damage may be another example. The progressive fibrotic process also involves the lungs, heart and gastrointestinal tract, and the latter was blamed for the marked hypomotility, rigidity and distension of the whole gastrointestinal canal, most prominently of the oesophagus, the colon and the rectum. However, Goldblatt et al. have revealed that the sera of SSc patients contain immunoglobulins that block the cholinergic agonist-evoked contraction of mouse colon strips in an apparently m3AChR-mediated fashion [32]. Later, Singh and co-workers confirmed these findings with the use of isolated rat colonic smooth muscle strips [33]. These results raise the possibility that a cholinergic dysfunction is also partly responsible for the dramatic loss of gastrointestinal motor activity in SSc.

FURTHER EXAMPLES OF ANTI-AUTONOMIC NEUROTRANSMITTER RECEPTOR ANTIBODIES

Nicotinic acetylcholine receptors localised in autonomic ganglia have been found to be a target of antibodies in patients with autonomic nervous system dysfunction. These symptoms range from orthostatic hypotension, gastrointestinal hypomotility and anhydrosis (loss of perspiration) to a debilitating collapse of autonomic regulation called pandysautonomia. These symptoms usually arise as a separate entity whereby autonomic dysfunction is not explained by the common causes of neuropathy including diabetes mellitus, toxic or degenerative causes. An autoimmune response targeting the autonomic ganglia has been proposed as the underlying mechanism, and in a large series of 15 000 patients tested, 155 were found to have anti-ganglionic nicotinic acetylcholine-receptor antibodies [34] Thirty percent of these patients had an underlying malignancy, and the presence of these antibodies was therefore considered as a paraneoplastic phenomenon. None of the SS- or systemic lupus erythematosus patients tested proved to be positive to these antibodies, and altogether there is only one SS-patient reported in the literature with ganglionic acetylcholine receptor antibody-positivity and an associated autoimmune autonomic ganglionopathy [35], thus these antibodies do not seem to play a major role in systemic autoimmune diseases, but their pathogenic role in cancer-associated or idiopathic autoimmune autonomic failure has been confirmed both in humans and animal models.

In addition to anti-m3AChR, antibodies with other specificities were also isolated from Chagas' disease patients' sera, namely anti-beta-1 adrenergic (anti-beta-1AR) antibodies [36]. Subsequently, these antibodies were also found in the sera of 25–90% of patients with dilated cardiomyopathy, a common and potentially fatal myocardium disease with multiple potential aetiologies and no established cure [37]. These antibodies were found to bind to the 2nd extracellular loop of the beta-1AR in most studies – similarly to anti-m3AChR – but the 1st extracellular loop was also proposed by other authors [38, 39]. Anti-beta-1AR antibodies are supposed to have an agonist or partial agonist activity on the receptor, leading to cAMP-accumulation, membrane-potential alterations that give rise to fatal arrhythmias, and myocyte degeneration or hypertrophy [40, 41]. The prevalence and potential patholphysiologic activity of anti-beta1-AR

receptors has nicely been reviewed by Nussinovitch and Shoenfeld [42].

CONCLUDING REMARKS

In sharp contrast to a wealth of interesting experimental data on the functional characteristics of all the three above-mentioned antibodies, it seems that the immunological detection of antireceptor antibodies is rather problematic. This obviously greatly impedes the convincing confirmation of the pathophysiological relevance of this mechanism in a number of serious diseases, and also hampers the assessment of their clinical importance and the development of routinely available tests for their detection. As conventional ELISA methods seem to fail in the hand of most of the groups, highly sophisticated techniques involving functional components have been introduced in specialised centres, including the assessment of receptor-mediated signaling by measuring cAMP by fluorescence-resonance energy transfer for the detection of anti-beta-1AR [43], and radioligand-binding assay on neuroblastoma cell line-derived nicotinic acetylcholine receptors by means of an Iodine^{125} labelled receptor agonist [44]. These problems indicate that he antigenic determinant of neurotransmitter receptors may be a particularly sensitive region in terms of antigenic structure, especially in macromolecules embedded in the lipid-bilayer of the plasma membrane with 7 molecular folds, or that the amount of antireceptor antibodies is so low in the serum that the standardization of the signal-noise parameters of immunodiagnostic tests may be accompanied by utmost difficulties.

The binding of these putative autoantibodies to their receptors may lead to either a receptor agonist or antagonist response. Either the deprivation of crucial neural stimuli or the recapitulation of the action of the physiological ligand in the form of a perpetual "overstimulation" will lead to the deviation of intracellular signal-transduction pathways, progressive subcellular alterations, and later tissue remodelling, and these will not only interfere with the function of these visceral organs but may eventually lead to permanent organ destruction.

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