Role of muscarinic-3 receptor antibody in systemic sclerosis: correlation with disease duration and effects of IVIG

Sumit Kumar,1 Jagmohan Singh,1 Ramalinga Kedika,1 Fabian Mendoza,2 Sergio A. Jimenez,3 Erik S. Blomain,4 Anthony J. DiMarino,1 Sidney Cohen,1 and Satish Rattan1

1Department of Medicine, Division of Gastroenterology and Hepatology, Thomas Jefferson University, Sidney Kimmel Medical College, Philadelphia, Pennsylvania; 2Division of Rheumatology, Thomas Jefferson University, Sidney Kimmel Medical College, Philadelphia, Pennsylvania; 3Jefferson Institute of Molecular Medicine and Scleroderma Center, Thomas Jefferson University, Philadelphia, Pennsylvania; and 4Department of Pharmacology and Experimental Therapeutics, Thomas Jefferson University, Sidney Kimmel Medical College, Philadelphia, Pennsylvania

Submitted 27 January 2016; accepted in final form 31 March 2016

Kumar S, Singh J, Kedika R, Mendoza F, Jimenez SA, Blomain ES, DiMarino AJ, Cohen S, Rattan S. Role of muscarinic-3 receptor antibody in systemic sclerosis: correlation with disease duration and effects of IVIG. Am J Physiol Gastrointest Liver Physiol 310: G1052–G1060, 2016. First published May 12, 2016; doi:10.1152/ajpgi.00034.2016.—Gastrointestinal dysmotility in systemic sclerosis (SSc) is associated with autoantibodies against muscarinic-3 receptor (M3-R). We investigated the temporal course of the site of action of these autoantibodies at the myenteric neurons (MN) vs. the smooth muscle (SM) M3-R in relation to disease duration, and determined the role of intravenous immunoglobulin (IVIG) in reversing these changes. Immunoglobulins purified from SSc patients (SScIgG) were used to test their differential binding to MN and SM (from rat colon) employing immunohistochemistry (IHC). Effect of SScIgG on neural and direct muscle contraction was determined by cholinergic nerve stimulation and bathenechol-induced SM contraction. Effects of IVIG and its antigen-binding fragment F(ab’2) on SScIgG binding were studied by enzyme-linked immunosorbent assay (ELISA) of rat colonic longitudinal SM myenteric plexus (LSMMP) lysate and to second extracellular loop peptide of M3-R (M3-RL2). SScIgG from all patients demonstrated significantly higher binding to MN than to SM. With progression of SSc duration, binding at MN and SM increased in a linear fashion with a correlation coefficient of 0.696 and 0.726, respectively (P < 0.05). SScIgG-mediated attenuation of neural and direct SM contraction also increased with disease duration. ELISA analysis revealed that IVIG and F(ab’2) significantly reduced SScIgG binding to LSMMP lysate and M3-RL2. Dysmotility in SSc occurs sequentially, beginning with SScIgG-induced blockage of cholinergic neurotransmission (neuropathy), which progresses to inhibition of acetylcholine action at the SM cell (myopathy). IVIG reverses this cholinergic dysfunction at the neural and myogenic receptors by anti-idiotypic neutralization of SScIgG.

scleroderma autoantibodies; muscarinic receptor; smooth muscle; myenteric neuron

SYSTEMIC SCLEROSIS (SSc) is a systemic autoimmune disease characterized by skin and internal organ fibrosis, vasculopathy, and immune dysregulation. Among the target organs affected by SSc, the gastrointestinal tract (GIT) is the most commonly affected internal organ. While dysmotility accounts for the vast majority of SSc-associated GIT symptoms, its pathogenesis is poorly understood (22, 26).

Recent advances in SSc pathogenesis have implicated immune dysregulation, vascular dysfunction, and fibrosis as the unifying mechanism of internal organ involvement (10). Among other factors, the lack of appropriate animal models reproducing gastrointestinal manifestations of SSc has limited our understanding of the pathophysiologic mechanism of dysmotility and has also hampered the development of new therapies (28).

Humoral immunity dysregulation has been recognized to play an important role in SSc pathogenesis. However, despite the fact that autoantibodies are present in more than 95% of patients with SSc, they were traditionally considered to be nonpathogenic. It is now hypothesized that anti-endothelial, anti-fibroblast, anti-MMP, and anti-fibrillin antibodies may be directly pathogenic in SSc (17). It has recently been demonstrated that IgG isolated from sera of SSc patients targets vascular smooth muscle cells and may be responsible for pulmonary hypertension (4). Similarly, research in the last decade has shown that gastrointestinal dysmotility in SSc may in part be related to functional autoantibodies (8, 11).

Earlier studies from our laboratory have demonstrated that gastrointestinal dysmotility in SSc is associated with circulating autoantibodies against the muscarinic-3 receptor (M3-R) (24, 25). These autoantibodies inhibited the contraction of smooth muscle cells (SMC) directly stimulated with a cholinergic agent and also blocked indirect muscle response induced by electric field neural stimulation suggesting cholinergic blockade by M3-R inactivation at neural and muscular levels. Of significant interest, the neural and myogenic effects of these autoantibodies were reproducibly abrogated by intravenous immunoglobulin (IVIG) strongly suggesting that the antibody could be removed from the receptor or could be neutralized in vitro (24, 25).

None of the earlier studies, however, examined the temporal sequence of neurogenic or myogenic site involvement, or investigated whether this involvement correlates with duration or severity of gastrointestinal SSc. Although treatment with IVIG has been studied in tight skin mouse models and in patients with cutaneous manifestations of SSc (21, 28), there are no data to indicate whether IVIG would be able to restore gastrointestinal dysfunction in SSc patients at different stages of the disease.

In this study, we tested the hypothesis that IgG from scleroderma patients (SScIgG) initially leads to neuropathy via inhibition of M3-R at the myenteric cholinergic neurons.
(MCN) which progresses to myopathy by inhibition of M3-R at the gastrointestinal SMC in the advanced stages of SSc.

The aims of the present study were 1) to investigate the effect of SScIgG at different stages of SSc (defined by duration of disease) by comparison of their sites of action at the MCN vs. the SMC; and 2) to determine the role of IVIG in reversing SSc gastrointestinal manifestations, and identify its mechanism of action.

MATERIALS AND METHODS

Subjects

Ten patients meeting the 2013 American College of Rheumatology criteria for the classification of SSc were selected retrospectively (29). Patients were included in the study if they had documented gastrointestinal symptoms attributable to SSc (determined by the UCLA SCTC GIT 2.0) along with typical esophageal manometric abnormalities. Patients on immunosuppressive and disease modifying drugs were excluded from the study. Patients were divided into two groups based on duration of SSc (skin manifestation was used to define the disease onset date). Patients with disease duration ≤ 15 years (180 mo) were placed in group I, while those in group II had a disease duration ≥ 16 years (192 mo).

Medical records for all participants were obtained to verify the diagnosis and to characterize the disease. We collected the following information about SSc patients: date of first diagnosis, extent of skin involvement (limited vs. diffuse), medication regimen, SSc specific autoantibody profile, gastrointestinal symptoms, esophageal manometry findings and UCLA SCTC GIT 2.0 score (18). The study was approved by the University’s Institutional Review Board.

Isolation and Purification of IgGs from SSc Patients and Normal Volunteers

Written informed consent was obtained for drawing blood samples. Total IgGs were purified from plasma of the 10 SSc patients (SScIgGs) and 2 normal volunteers (NlgGs) by the use of biocompatible polypropylene columns packed with 5 ml of protein G-Sepharose from genetically modified protein G lacking albumin affinity and with high binding capacity for human IgG (HI-trap protein G HP-GE Healthcare, Pittsburgh, PA) (13).

Experiments Using Intact Rat Colon Smooth Muscle (SM) Strips

Colonic smooth muscle strip preparations and isometric tension recording. Male Sprague-Dawley rats (300–350 g) were euthanized by decapitation, and the lower portion of the colon was removed surgically and transferred to oxygenated (95% O2 + 5% CO2) Krebs physiological solution (KPS) at 37°C. The composition of KPS (in mmol/l) was as follows: 118.07 NaCl, 4.69 KCl, 2.52 CaCl2, 1.16 MgSO4, 1.01 NaH2PO4, 25 NaHCO3, and 11.1 glucose. The SM strips (7–10 mm long) were prepared from the circular SM layer of the colon.

The SM strips were then transferred to 2-ml organ baths containing oxygenated KPS. Isometric tension was monitored by use of force transducers (FORT10, WPI, Sarasota, FL) and Chart 4.1.2 via PowerLab/8SP data-acquisition system (AD Instruments, Colorado Springs, CO). Each SM strip was initially stretched to a tension of 1.0 g followed by 90 min of equilibration. The basal tone in each SM strip was determined at the end of the experiment by the administration of Ca2+-free (0Ca2++) KPS. The experimental protocol of the study was approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Direct muscle stimulation with bethanechol (BeCh). The responses of the colonic SM strips to BeCh (10−7 to 10−3 M) were quantified before and after NlgG (1 mg/ml), SScIgG (1 mg/ml), IVIG (10 mg/ml), and IVIG (10 mg/ml) + SScIgG (1 mg/ml) after incubation in the muscle bath for 15 min. To determine the selectivity of the action of SScIgG on M3-R inactivation, we compared the effects of K+ depolarization by KCl (2.5 to 40 mM). These contractile responses were calculated and plotted as percentage of maximal contraction by 10−4 M BeCh.

Cholinergic nerve stimulation experiments. To determine the effects of cholinergic nerve stimulation, electrical field stimulation (EFS; 10 V, 0.5–20 Hz, 4-s train, each pulse of 0.5 ms) was delivered using a Grass stimulator (model S88; Grass Instruments, Co, Quincy, MA). The EFS responses (percentage maximal increase in the basal activity) were quantified before and after NlgG (1 mg/ml), SScIgG (1 mg/ml), IVIG (10 mg/ml), and IVIG (10 mg/ml) + SScIgG (1 mg/ml).

Acetylcholine (ACh) measurements. To measure the release of ACh in response to EFS, we determined the effect of 30-s train of EFS (10 V, 5 Hz, each pulse of 0.5-ms duration). The muscle bath perfusates were collected in the basal state, and before and after NlgG (1 mg/ml), SScIgG (1 mg/ml), IVIG (10 mg/ml), and IVIG (10 mg/ml) + SScIgG (1 mg/ml).

Immunohistochemistry (IHC) of Rat Colon

Whole mount and sections. Rats were euthanized by decapitation and colon was removed immediately and cleaned with 4% paraformaldehyde and kept in it overnight. Paraformaldehyde was then replaced with 70% ethanol and tissues were sent for embedding and sectioning to Thomas Jefferson University Histopathology Core facility. Sections were deparaffinized by keeping them for 30 min at 70°C and washed two times (10 min each wash) in xylene. Sections were hydrated in 2 changes of 100% ethanol for 5 min each, followed by 2 changes of 95% ethanol, 1 change of 80% ethanol, and 2 changes of 70% ethanol for 3 min each. Sections were then rinsed in 2 changes of distilled water for 5 min each. Antigen retrieval was done in Tris-buffered saline containing Tween-20 (0.05 M TBS, 0.05% Tween-20, pH 9.0) in a pressure cooker for 30 min. Slides were washed twice with distilled water and stained with cuprolinic blue for 30 min (15). Slides were then washed with distilled water twice and blocked with donkey serum in PBST for 1 h. SScIgG and Anti-M3-R against rat were added on the sections in PBST and kept in humidified chamber at 4°C for overnight incubation. Then, the slides were washed with PBST and stained with anti-human IgG and Anti-goat IgG in PBST buffer and incubated at room temperature (RT) for 1 h and washed three times with PBST and fixed with Vectashield mounting media (Burlingame, CA) and viewed under confocal microscope (Carl Zeiss, Germany) as well as an Evos fluorescent microscope (Life technologies), and photographs were taken and analyzed with ImageJ2 (NIH).

Immunofluorescence intensity calculation. Immunofluorescence intensity (IFI) was calculated by using ImageJ2 (NIH). Areas of interest from the images were selected corresponding to the myenteric plexus and smooth muscle, and intensity surface plots were plotted and the intensity (IFI) was calculated by using ImageJ2 plugins and plotted as bar graph in prism software.

Isolation of longitudinal smooth muscle-myenteric plexus (LSMMP) layer. Rat colon was removed proximal to the sigmoid colon and divided into 3- to 4-cm segments. The lumen of these segments was flushed with KPS. These segments were then stretched over a glass rod and the mesentery carefully removed. The longitudinal muscle layer with adherent myenteric plexus was separated from...
the underlying circular muscle layer by gently stroking tangentially away from the mesenteric attachment with a cotton-tipped swab and peeling away the mucosal, submucosal and circular SM layers (2). LSMMP was used for IHC, and its lysates prepared under fresh conditions were used for ELISA.

**Enzyme-linked immunosorbent assay (ELISA).** To determine the binding affinity of SScIgG to the M3 R we performed ELISA studies with M3-RL2 synthetic peptide and LSMMP lysate; 400 µg/ml rat colonic LSMMP lysate and 400 µg/ml of a peptide corresponding to the M3-RL2 (KRTVPQGECFIQFLSEPITFTGTAI, amino acids 213–237) were separately dissolved in carbonate buffer and adsorbed onto separate multwell plates used for ELISA. Between 5 and 40 nM each of SScIgG, IVIG, antigen binding fragment of IVIG [F(ab')2] and SScIgG + IVIG were added to these multwell plates and incubated at 37°C for 1 h. Plates were washed three times with DPBST and incubated for 1 h at RT with anti-human-HRP-conjugated secondary antibodies for SScIgG, or IVIG, washed three times with DPBST, and 100 µl of TMB substrate was added and kept for 15 min at RT. Then, 100 µl of Stop solution was added to each well, and absorbance was recorded at 450 nm with ELISA reader.

### Drugs and Chemicals

Bethanechol, KCl, and M3-R antibody were purchased from Sigma Aldrich (St. Louis, MO). M3-R loop-2 (M3-RL2) was purchased from Peptide 2.0 (Chantilly, VA). IVIG and its pepsin-derived antigen-binding fragment F(ab')2 were prepared as previously described (16) and were obtained from CSL Behring (King of Prussia, PA).

### Statistical Analysis

Data are presented as means ± SE of multiple experiments. *P* values < 0.05 were considered statistically significant. The concentration-response curves were fitted by nonlinear regression, and comparisons were made using unpaired Student’s *t*-test, or ANOVA, using the computer software Prism (GraphPad Software, San Diego, CA). Pearson’s coefficient was determined to assess correlation between binding and disease duration.

### RESULTS

#### Characteristics of Study Participants

All patients were females aged between 38 and 73 yr (Table 1). Among all patients, 6 had diffuse cutaneous involvement, and 4 had limited disease. The mean ± SD disease duration of patients in group I was 141.6 ± 35.4 mo, while that of patients in group II was 290.4 ± 95.4 mo. Their gastrointestinal symptom score as determined by the UCLA SCTC GIT 2.0 questionnaire ranged from 0.75 to 15.1. No significant correlation was found among various SSc specific autoantibodies and gastrointestinal disease.

#### Baseline characteristics of patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, yr</th>
<th>Disease Duration, mo</th>
<th>SSc Subtype</th>
<th>UCLA GIT 2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71</td>
<td>108</td>
<td>Limited</td>
<td>3.47</td>
</tr>
<tr>
<td>2</td>
<td>73</td>
<td>120</td>
<td>Limited</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>120</td>
<td>Limited</td>
<td>1.85</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>180</td>
<td>Diffuse</td>
<td>2.74</td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>180</td>
<td>Diffuse</td>
<td>2.04</td>
</tr>
<tr>
<td>6</td>
<td>49</td>
<td>192</td>
<td>Diffuse</td>
<td>0.75</td>
</tr>
<tr>
<td>7</td>
<td>62</td>
<td>240</td>
<td>Limited</td>
<td>2.25</td>
</tr>
<tr>
<td>8</td>
<td>61</td>
<td>240</td>
<td>Diffuse</td>
<td>15.1</td>
</tr>
<tr>
<td>9</td>
<td>67</td>
<td>360</td>
<td>Diffuse</td>
<td>2.49</td>
</tr>
<tr>
<td>10</td>
<td>64</td>
<td>420</td>
<td>Diffuse</td>
<td>4.72</td>
</tr>
</tbody>
</table>

**Binding of SScIgG to Myenteric Plexus (MP) and Smooth Muscle (SM)**

Myenteric plexus in whole mount rat colonic sections was identified by selective staining with neuronal dye cuprolinic blue. SScIgG from all ten patients demonstrated positive staining with rat colonic myenteric plexus and smooth muscle (Fig. 1A). Immunofluorescence intensity (IFI) analysis revealed that the binding intensity of SScIgG to myenteric plexus was significantly higher (IFI of SScIgG bound to MP was considered as 100%) in contrast to smooth muscle immunofluorescence intensity, which was <50% (Fig. 1B; *P* < 0.05; *n* = 3). These data reveal that SScIgG binds at two sites in the rat colon: myenteric plexus and the smooth muscle, but with higher binding intensity to the former.

**Colocalization of SScIgG and M3-R on MP and SM**

Data show that SScIgG binds specifically to the M3-R on rat colonic SM and myenteric plexus (Fig. 1C). IFI analysis showed a significant colocalization of M3-R and SScIgG binding at the colonic myenteric plexus and smooth muscle (correlation coefficient 0.70 and 0.85, respectively) (Fig. 1D; *P* < 0.05; *n* = 3). This binding of SScIgG at the same position corresponding with M3-R antibody binding suggests that SScIgG binds at the M3-R on both the smooth muscle and myenteric plexus.

**Disease Duration-Dependent Binding of SScIgG at MP and SM**

Data reveal that IgG from a patient in group I (disease duration: 120 mo) had significantly higher binding at myenteric plexus compared with binding at the smooth muscle (IFI 0.36 vs. 0.27, respectively) (Fig. 2A; *P* < 0.05; *n* = 3). In contrast, IgG from a patient in group II (disease duration: 420 mo) revealed significantly higher binding at both the myenteric plexus and smooth muscle (IFI 0.53 vs. 0.50, respectively), with the binding intensity at the latter almost approaching the binding intensity at the myenteric plexus (Fig. 2A; *P* < 0.05; *n* = 3).

The IFI of SScIgG binding, at myenteric plexus and smooth muscle from all patients when plotted against duration of disease, revealed a strong positive correlation coefficient of *R* = 0.7, *P* < 0.05 and *R* = 0.8, *P* < 0.05, respectively (Fig. 2B). Data suggest that binding of SScIgG at myenteric plexus and smooth muscle increases with the duration of disease in a linear fashion accounting for the progressive nature of the disease.

**Effect of IVIG on SScIgG Binding**

IVIG reversed the binding of SScIgG at both the myenteric plexus and smooth muscle as evidenced by the decrease in IFI (Fig. 2, C and D; *P* < 0.05; *n* = 3). This reversal effect was consistently observed with SScIgG from all ten patients.

**Effect of SScIgG on Cholinergic Nerve Stimulation in Intact Rat Colonic SM**

EFS caused a frequency-dependent increase in the contraction of the colonic smooth muscle. The data further revealed that SScIgG (1 mg/ml) and not IVIG (10 mg/ml) by itself, caused inhibition of EFS-induced smooth muscle contraction. Moreover SScIgG from a patient in group II (disease duration: 420 mo) nearly obliterated the EFS-induced response to 80%,
compared with the effect of the SScIgG from a patient in group I (disease duration: 120 mo) which caused only 30% inhibition of EFS response (Fig. 3, A and B; \( P < 0.05; n = 4 \)). This effect of SScIgG was partially reversed when it was administered premixed with IVIG (Fig. 3, A and B; \( P < 0.05; n = 4 \)).

These findings signify that with progression of disease duration, greater proportion of IgG molecules within the SScIgG pool recognizes the target epitope (M3-R) leading to progression of cholinergic neuropathy.

**Effect of SScIgG on EFS-Evoked ACh Release in Intact Rat Colonic SM**

Measurement of ACh release from colonic smooth muscle stimulation revealed that in control experiments (in the presence of normal IgG) EFS (10 V, 5 Hz, 0.5 ms, 30-s train) caused a significant increase in ACh release, which was attenuated by \( 0Ca^{2+} \) but remained unaffected by IVIG. In addition, SScIgG caused a significant decrease (*\( P < 0.05 \); Fig. 3C) in ACh release. Notably SScIgG from patients in group II caused further significant decrease (*\( P < 0.05 \); Fig. 3C) in ACh release compared with SScIgG from patients in Group I, validating the progression of cholinergic neuropathy with advancement of disease duration. The suppressant effect of SScIgG (from patients in both groups) on ACh release was reversed by IVIG (Fig. 3C; \( P < 0.05; n = 3 \)), which by itself had no significant effects on basal release of ACh. These data suggest that IVIG could potentially reverse SScIgG-mediated cholinergic dysfunction.

**Effect of SScIgG on Direct Colonic Smooth Muscle Contraction by Bethanechol (BeCh)**

BeCh caused a concentration-dependent increase in contraction of the colon smooth muscle strips that was attenuated by
SScIgG. KCl also caused concentration-dependent increase in colon smooth muscle contraction that was not affected by SScIgG (Fig. 4, A and B; \( P < 0.05; n = 4 \)) suggesting the selectivity of the suppressant effects of SScIgG on M3-R activation in the colon.

In these experiments the effect of SScIgG (1 mg/ml) from a patient in group I (disease duration: 120 mo) was compared with that of SScIgG (1 mg/ml) from a patient in group II (disease duration: 420 mo). SScIgG from the former patient with disease of less duration attenuated BeCh response to only 70% of control (Fig. 4B; \( P < 0.05; n = 4 \)) whereas SScIgG from patient in group II led to much greater attenuation of BeCh response to 50% of maximal contraction (Fig. 4C; \( P < 0.05; n = 4 \)). [Maximal contraction with BeCh (10\(^{-4}\) mol/l) was regarded as 100% contraction.]

The attenuation of smooth muscle contraction induced by SScIgG from both patients was significantly reversed by pre-treatment of the smooth muscle strips with 10 mg/ml IVIG (Fig. 4, B and C; \( P < 0.05, n = 4 \)). IVIG by itself had no significant effect on BeCh-induced smooth muscle contraction.

Assessment of SScIgG Binding to M3-R and Influence of Complete IVIG And F(ab')\(_2\) Using Synthetic M3-RL2 Peptide and LSMMP Lysate

In these experiments, we used multiwell plates (used for ELISA) preadsorbed with 400 pg/ml of M3-RL2 or 400 \( \mu \)g/ml of LSMMP lysate and determined the OD after incubation with varying concentrations of SScIgGs. Data show that SScIgG (and not NIgG) binds to LSMMP significantly in a concentration-dependent manner with the maximal OD at 40 nM of SScIgG as 0.532 (Fig. 5A; \( *P < 0.05; n = 4 \)). Pretreatment of the SScIgG with IVIG (SScIgG/IVIG) and its F(ab')\(_2\) fragment [SScIgG + F(ab')\(_2\)] caused a significant decrease in the binding of the SScIgG to the LSMMP lysate as reflected by a decrease in the OD (to 0.148 and 0.104, respectively) (Fig. 5A; \( P < 0.05; n = 4 \)). These data suggest that the LSMMP has M3-R domains that bind selectively with the SScIgGs.

ELISA with M3-RL2 revealed similar results that showed a concentration-dependent increase in M3-R binding with...
SScIgG (Fig. 5B; *P < 0.05; n = 4) and not with NIgG (P > 0.05). Maximal OD at 40 nM of SScIgG was 0.54 (Fig. 5B; P > 0.05; n = 4). Pretreatment of the SScIgG with IVIG (SScIgG+IVIG) and its F(ab’)_2 fragment [SScIgG+F(ab’)_2] caused a significant and concentration-dependent decrease in the OD (P < 0.05; n = 4).

When the OD obtained with SScIgG (40 nM) binding to M3-RL2 from all patients was plotted against their duration of disease a selective and disease duration-dependent increase in the binding was observed. This binding was attenuated significantly by IVIG and also by its antigen-binding fragment F(ab’)_2 suggesting the presence of anti-idiotypic antibodies in IVIG that neutralize the pathological activity of SScIgGs (Fig. 5C; P < 0.05).

DISCUSSION

In continuation from our previous work on the pathogenesis of gastrointestinal dysmotility in SSc, the current study demonstrates 1) SScIgGs from patients with disease duration of less than 15 years display high affinity inhibition of M3-R at myenteric cholinergic neurons (MCN); 2) further progression of disease duration leads to combined inhibition of M3-R at the MCN and smooth muscle (SM); and 3) IVIG attenuates the
binding of these autoantibodies at the MCN and SM receptors by anti-idiotypic neutralization.

Evidence for neuropathy in gastrointestinal SSc comes from two landmark studies in the 1970s. First, Cohen et al. (5) demonstrated that the esophageal smooth muscle responded to direct acting agent methacholine but not to indirectly acting edrophonium. (Edrophonium increases the levels of ACh by preventing its breakdown by acetylcholinesterase whereas methacholine, similar to ACh, is a muscarinic receptor agonist.) Subsequently, DiMarino et al. (7) showed that duodenal myoelectric activity was intact but there was an abnormality in intestinal activation by mechanical and hormonal stimuli in SSc patients. These data collectively suggest an early neuropathic phase characterized by dysregulation of ACh release from the myenteric cholinergic neurons. Further evidence of neuropathy in SSc is supported by studies demonstrating impaired anal sensation and rectoanal inhibitory reflex in this subset of patients (14).

Multiple mechanisms were proposed to explain the mechanism of neuropathy in SSc but none could gather much scientific attention until Goldblatt et al. (11) demonstrated that sera of SSc patients contained antibodies that could inhibit cholinergic-mediated contraction of the mouse colon. The strongest evidence for autoantibody-mediated dysmotility can be drawn from a study that showed that SSc patients develop manometric abnormalities in the absence of histopathological changes in the esophageal musculature (27). Studies from our laboratory confirmed subsequently that autoantibodies isolated from SSc patients inhibit direct and indirect (neurally mediated) rat colonic and internal anal sphincter smooth muscle contraction (24).

The present studies provide evidence in favor of the temporal course of SScIgG binding to the neural and myogenic M3-R. Immunohistochemical data reveal that binding of SScIgG at the myenteric cholinergic neurons was higher in patients early in the course of their disease and further increased over time. Most notably, binding to the SM increased over time as well. These data demonstrate a positive correlation between the binding intensity of SScIgG to the myenteric neurons and smooth muscle with disease duration. Thus, greater the duration of the disease, the greater is the binding accounting for the progressive nature of GIT involvement in SSc. Similar results were shown in a study wherein esophageal stiffness and impaired muscle function on manometry correlated with disease duration (12).

Results of functional studies further reiterate the higher affinity of SScIgG to the MCN earlier and later to smooth muscle with progression of the disease. This progressive effect of SScIgGs at the M3-R located on myenteric cholinergic neurons was shown by the marked decline in EFS-induced colonic smooth muscle contraction and actual release of ACh following pretreatment of the smooth muscle strips with SScIgG from patients from group II compared with patients from group I. More importantly, SScIgGs from patients with significantly advanced duration of disease in group II inhibited BeCh-induced contraction of the rat colonic SM to a greater extent compared with group I, suggesting the targeting of smooth muscle M3-R (myopathy) with disease progression.

M3-R-specific ELISA studies revealed that SScIgGs cause a selective and disease duration-dependent increase in the binding to the M3-RL2 peptide and whole M3-R in LSMMP lysates. Although the exact reason for such an increase in affinity of SScIgGs toward MCN as well as SM (observed during ELISA, and IFI studies as discussed above) is not known, a few possibilities exist: Firstly, a change in the epitope of M3-R that is recognized by SScIgG (owing to structural or anatomical changes in the target tissues possibly caused by edema or inflammation) as SSc progresses. Secondly, a phenomenon of affinity maturation of the antibody response (6, 9, 20), with disease duration (12).

Present studies provide further evidence that IVIG reverses SScIgG-induced M3-R inactivation at both the neural and myogenic sites. Evidence for the effects of IVIG comes first from IHC studies showing significant decrease in M3-R
binding intensity following SScIgG, at both the myenteric cholinergic neurons and smooth muscle. Second, IVIG reverses both the SScIgG-induced attenuation of BeCh and EFS-stimulated colonic muscle contraction. Finally, IVIG attenuates SScIgG-induced increase in binding intensity to the M3-R when multiwell plates are preadsorbed with LSMMP lysate or M3-RL2 peptide.

In our previous study we had demonstrated that IVIG produces its effect by directly competing with pathogenic SScIgGs (24, 25). The three main mechanisms that are likely to involve direct competition with pathologic autoantibodies are anti-idiotypic binding, FcRn saturation, and complement scavenging (3, 23). Our findings that F(ab')2 fragments of the IVIG have the same effect as IVIG in vitro (ELISA binding studies to the M3-RL2 peptide and whole M3-R in LSMMP) suggest the presence of anti-idiotypic antibodies in IVIG that block the activity of pathogenic SScIgGs (3).

Currently the treatment of gastrointestinal disease in SSc is symptomatic and ineffective (19). In this regard IVIG offers hope for treatment of SSc-associated gastrointestinal motility disorders. A recent study focusing on the effect of IVIG in SSc patients reported no significant worsening in gastrointestinal symptom score at 1 yr follow up as a secondary end point (21). However, the exact mechanism, and the role of other pathways in the therapeutic efficiency and efficacy of IVIG, remain to be determined.

SSc is a heterogeneous disease in which internal organ involvement and outcomes vary considerably from patient to patient. There is a subset of patients who develop rapidly progressive debilitating gastrointestinal symptoms within a few years of diagnosis. Whether this subset of patients exhibit neuropathy followed by myopathy, remains to be determined. Moreover, autoantibody-associated dysmotility does not explain the mechanism of fibrosis seen in the gastrointestinal tract and most importantly does not correlate with the symptomatology of gastrointestinal disease (determined by the UCLA SCTC GIT 2.0). Another limitation of the present study is sample size in terms of limited number of patients. Despite these limitations, the results of our study are novel and should be followed up with a multicenter study involving a large number of SSc patients at different stages of the disease.

In conclusion we propose that the pathophysiological changes of the gut in SSc occur in a staged process beginning with neuropathy and progressing to myopathy (see Fig. 6). Initially, circulating M3-R autoantibodies block cholinergic neurotransmission via inhibition of ACh release at the MCN (neuropathic damage) and later lead to myopathy via inhibition of ACh action at the gastrointestinal smooth muscle cell proper. We further suggest that SSc-associated intestinal dysfunction at both the neuropathic and myopathic stages may be potentially reversible with IVIG.

**Fig. 6.** Proposed pathogenesis of gastrointestinal dysmotility in SSc suggests that SScIgGs initially block cholinergic neurotransmission by inhibition of ACh release by the MCN (neuropathy). With progression of the disease, SScIgGs lead to additional myopathy via inhibition of ACh action at the gastrointestinal smooth muscle proper (myopathy). This is followed by the last stage of intestinal fibrosis by yet unknown mechanisms. Dysmotility at the neuropathic and myopathic stages may be potentially reversible with IVIG, before smooth muscle fibrosis/atrophy ensue.
GRANTS

The work was supported by Grant Number RO1-DK-035385 from the National Institutes of Diabetes and Kidney Diseases, an industrial support from CSL Behring, King of Prussia, PA, and an institutional grant from Thomas Jefferson University. E. S. Blomain is the recipient of the Ruth L. Kirschstein National Research Service Award for Individual Predoctoral MD/PhD Fellows (1 F30 CA180500) from the National Institutes of Health.

AUTHOR CONTRIBUTIONS


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES