Antimyenteric Neuronal Antibodies in Scleroderma

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Abstract

The pathogenesis of gastrointestinal (GI) dysmotility in scleroderma is incompletely understood, although previous studies have proposed a neuropathic mechanism. We studied patients with scleroderma as compared with other connective tissue disease patients and normal controls for the presence of circulating antibodies to myenteric neurons. Serial dilutions of sera were overlaid on rat intestine, double-labeled with antineurofilament antibody as a myenteric plexus marker, and imaged using indirect immunofluorescence techniques. High titer sera (≥ 1:50) from 19 out of 41 scleroderma patients stained myenteric neurons, whereas none of 22 normals or 5 patients with idiopathic GI dysmotility were positive. Although 6 out of 20 SLE and 6 out of 10 mixed connective tissue disease patients' sera stained myenteric plexus neurons, when positive sera were absorbed with calf thymus extract to remove antinuclear antibody, 15 scleroderma sera, 0 SLE, and 2 mixed connective tissue disease patients retained positive staining of myenteric neurons. Western blotting using actin and neuronal intermediate filament preparations failed to show immunoreactivity with scleroderma sera containing antimyenteric neuronal antibodies. Paraneoplastic sera associated with GI dysmotility stained myenteric neurons in a different pattern than seen with scleroderma sera. A positive correlation between the presence of Raynaud's phenomenon and antimyenteric neuronal antibodies was observed in scleroderma patients. Our results indicate that IgG antibodies reacting with myenteric neurons are present in many patients with scleroderma. Although the neuronal antigen has not yet been identified, the presence of myenteric neuronal antibodies in patients with GI dysmotility and scleroderma suggests a neuropathic process. (*J. Clin. Invest.* 1994. 94:761–770.)

Key words: myenteric plexus • cytoskeletal proteins • scleroderma, systemic • gastrointestinal motility

Introduction

Autoantibodies have long been recognized in association with scleroderma but have not been related to the pathogenesis of the disease. Several novel autoantibodies present in serum of scleroderma patients such as anticentromere or anti–topoisomerase I (Scl-70) antibodies have been correlated with disease presentation (1–3). Although specific disease manifestations vary in individual patients, various subgroups have been recognized, such as the CREST variant (**C**alcinosis, **R**aynaud's phenomenon, **E**sophageal dysmotility, **S**clerodactyly, and **T**elangiectasia)[1] which is often associated with the presence of anticentromere antibodies. Several novel autoantibodies found in scleroderma, including anti–RNA polymerase I, antifibrillarin, and anti–PM-Scl, are directed at antigens present in the nucleolus depending on cell function or cycle.

Gastrointestinal (GI) dysmotility can occur throughout the clinical spectrum of scleroderma diseases and sometimes occurs as the presenting manifestation. Although esophageal dysfunction is most often recognized, smooth muscle function throughout the entire GI tract may be affected. The pathogenesis of esophageal dysfunction and GI dysmotility in scleroderma is poorly understood. Association of Raynaud's phenomenon with GI dysmotility (4) suggests a common mechanism, such as autonomic dysfunction or microvascular pathology. Some patients, however, have been noted to have severe GI dysmotility without evidence for cutaneous disease, increase in esophageal fibrosis or connective tissue, or other manifestations most often recognized as scleroderma (5, 6). Moreover, previous studies (7–9) have proposed a neuropathic mechanism to account for the GI dysmotility seen in these patients.

Paraneoplastic syndromes with GI dysmotility have been shown to be associated with the presence of circulating autoantibodies to the myenteric plexus (10–12) and may actually herald the appearance of the tumor. Although a distinct autoantibody has not been described in association with GI dysmotility in scleroderma, the possible involvement of autoantibody being directly related has never been explored carefully. Therefore, we undertook the present study for evidence of circulating antimyenteric neuronal antibodies in scleroderma patients.

Methods

Patients. 41 patients with scleroderma or a scleroderma-like connective tissue disorder who had positive antinuclear antibodies (ANAs) were prospectively selected from patients seen in the Rheumatology and Gastroenterology Clinics at the University of Florida Shands Hospital and at Arthritis Associates clinic in Gainesville, FL. Many of these patients had clinical, functional, or manometric evidence for GI dysmotility involving the esophagus and upper GI tract. The treating physician determined the appropriate GI tract workup in each patient. Clinical profiles of all patients studied are shown in Table I. Nine patients classified as definite scleroderma had the diagnosis confirmed by histo-

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1. *Abbreviations used in this paper:* ANA, antinuclear antibody; ANNA-1, antineuronal nuclear antibody, type 1; CREST, calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia; GI, gastrointestinal; MCTD, mixed connective tissue disease; NF-H, high-molecular-weight neurofilament protein; RNP, ribonucleoprotein.

Table I. Clinical Characteristics of Scleroderma Patients

Diagnosis	Age	Raynaud's	Skin disease	GI symptoms and study profiles	Other
Diffuse scleroderma					
Patient 1	62	+	+	Esophagram: poor contractions. Diarrhea, cramping, constipation	Severe pulmonary hypertension
Patient 2	47	+	+	Reflux symptoms	Pulmonary hypertension
Patient 3	18	+	+ (Biopsy proven)	Reflux and dyspepsia	Pulmonary hypertension
Patient 4	67	+	+ (Biopsy proven)	Reflux symptoms and diarrhea	Pulmonary hypertension Myositis
Patient 5	37	+	+	Denies symptoms	—
Patient 6	64	n/a*	+	Reflux symptoms and diarrhea	—
Limited scleroderma					
Patient 7	83	+	+	Denies symptoms	Pulmonary fibrosis Cardiomyopathy
Patient 8	76	+	−	Denies symptoms	Pulmonary fibrosis, severe
Patient 9	59	+	+	Denies symptoms	—
Patient 10	68	+	+ (Biopsy proven)	Small bowel manometry: poorly formed, low amplitude contractions	Restrictive lung disease
Patient 11	65	+	+	Bacterial overgrowth. Recurrent esophageal stricture. Esophageal manometry: aperistalsis, normal (17.7 mmHg) LES‡	Pulmonary hypertension
Patient 12	56	+	+	Reflux symptoms. Barium esophagram: dilated with decreased peristalsis	—
Patient 13	67	n/a	+	Constipation	Pulmonary fibrosis
Patient 14	42	+	+	Refused workup. Subjective dysphagia	—
Patient 15	64	+	+	Reflux symptoms	—
Patient 16	76	+	+	Reflux symptoms with esophagitis	—
Patient 17	52	+	+	Barium esophagram: decreased peristalsis, esophageal dilation, lower esophageal stricture. Dilated small bowel	—
Patient 18	71	+	+	Diarrhea, bloating	—
Patient 19	38	+	+	Dysphagia, constipation	—
Patient 20	58	−	+ (Biopsy proven)	Denies symptoms	—
Patient 21	69	−	+	Dysphagia, reflux symptoms, early satiety, diarrhea	—
CREST					
Patient 22	59	+	+ (Biopsy proven)	Gastric emptying study: 25.9% at 2 h (normal 70%). Esophagram: no peristalsis lower 2/3. Biopsies esophagus, stomach, and duodenum: normal architecture	Distal sensory neuropathy
Patient 23	38	+	+	Reflux symptoms. Esophageal manometry: LES 6 mmHg, occasional aperistalsis. Gastric emptying study: $t_{1/2}$ = 100 min (normal < 75 min). Diarrhea	Restrictive lung disease
Patient 24	58	+	+	Barium esophagram: lower esophageal stricture, good peristalsis	—
Patient 25	54	+	+	Esophagoscopy: esophageal ulcers	—
Patient 26	41	+	+	Denies symptoms	—
Patient 27	71	−	+	Barium esophagram: dilated esophagus. Reflux symptoms	Myositis

Table I. (Continued)

Diagnosis	Age	Raynaud's	Skin disease	GI symptoms and study profiles	Other
Morphea					
Patient 28	63	+	+ (Biopsy proven)	Reflux symptoms. Chronic abdominal pain (?pancreatitis)	—
Patient 29	52	−	+ (Biopsy proven)	Severe reflux. Gastric emptying study: $t_{1/2}$ = 110 min (normal < 75 min)	Thyroiditis
Patient 30	57	−	+ (Biopsy proven)	Esophageal manometry: low amplitude contractions with occasional aperistalsis. LES = 10 mmHg	—
Overlap (polymyositis-limited scleroderma)					
Patient 31	69	+	+	Denies symptoms	Thyroiditis, myositis
Overlap (SLE-limited scleroderma)					
Patient 32	29	+	+	Bacterial overgrowth by D-xylose. Gastric emptying study: $t_{1/2}$ = 90 min, with prompt emptying of stomach after intravenous erythromycin	Pulmonary fibrosis, diabetes, proteinuria, pericarditis
Overlap (SLE-limited scleroderma)					
Patient 33	59	+	+	Dysphagia	Chorea, thrombocytopenia, antiphospholipid syndrome
Overlap (SLE-morphea)					
Patient 34	21	+	+ (Biopsy proven)	Denies symptoms	—
Overlap (polymyositis-limited scleroderma)					
Patient 35	64	+	+	Denies symptoms	Myositis
Undifferentiated connective tissue disease					
Patient 36	41	+	−	Esophageal manometry: peristaltic and aperistaltic contractile activity of low, repetitive amplitude, occasional drop out of amplitude. LES = 5 mmHg	—
Patient 37	72	−	−	Gastroesophageal reflux, gastritis	—
Patient 38	36	−	−	Denies symptoms	Polyarthritis
Patient 39	52	−	−	Postprandial bloating and reflux. Delayed gastric emptying	—
Patient 40	50	+	−	Recurrent esophageal stricture	Autoimmune chronic active hepatitis
Patient 41	22	+	−	Denies symptoms	—

* n/a, accurate information not available. ‡ LES, lower esophageal sphincter pressure (mmHg), normal > 10–12 mmHg.

logic examinations of skin biopsies. Among all patients, six had diffuse scleroderma involving face, extremities, neck, and trunk. 15 had limited scleroderma (obvious skin thickening limited to the distal extremities and face, or to a localized area of the skin), 6 had CREST syndrome, and 3 had morphea (localized scleroderma without disseminated disease). Five patients had scleroderma overlap syndromes, two of which had scleroderma associated with myositis, and three with scleroderma and features of SLE. Six had undifferentiated connective tissue disease syndromes (Raynaud's phenomenon, GI dysmotility, and strongly positive ANAs, but without a clearly definable single connective tissue disorder). Charts were reviewed, and patients were interviewed for pertinent clinical data including Raynaud's phenomenon, skin disease, biopsy reports, therapy, concurrent illnesses, and history of GI motility disorders. Sera from 22 normal laboratory personnel and hospital staff without history of scleroderma or GI dysmotility and 5 serum samples from patients with idiopathic GI dysmotility (no history of autoimmune diseases, cancer, diabetes, or thyroid disease) were obtained for comparison. Two serum samples from patients with paraneoplastic syndrome known to contain type 1 antineuronal nuclear antibodies (ANNA-1, also known as "anti-Hu") (10–12) with antimyenteric neuronal immunolocalization (courtesy of Dr. Vanda Lennon, Mayo Clinic, Rochester, MN) were obtained as positive controls.

Other connective tissue disease patient controls. Sera from several groups of patients with other connective tissue diseases were also studied in parallel. These included 20 patients with rheumatoid arthritis and 20 patients with SLE who satisfied American College of Rheumatology criteria for definite disease (13, 14). An additional group of 10 patients with mixed connective tissue disease (MCTD) but without clinical fea-

tures of scleroderma (Raynaud's phenomenon or GI dysmotility) was also studied.

Serology. Serum samples from patients were characterized in the laboratory for fluorescent ANA using mouse kidney sections and Hep-2 cell substrates, for associated serology using MOLT-4 (T-lymphocyte cell line) extracts for Western blots, and by double diffusion analysis for Scl-70 and ribonucleoprotein (RNP) antibody (15). Three patients' sera were not available for such further characterization.

Tissue and indirect immunofluorescence. Healthy adult Harlan Sprague-Dawley rats were anesthetized and decapitated, and distal esophagus, stomach, small intestine, and colon were removed. Studies were approved by the University of Florida Animal Use Committee. Tissues were frozen in super-cooled isopentane at −80°C and mounted, and 5–8-μm cryostat sections were made longitudinally on a freezing microtome (model 327; International Equipment Co., Needham Heights, MA). Sections of longitudinal muscle and myenteric plexi were mounted on glass slides, fixed in acetone at −20°C for 5 min, and air-dried overnight before antibody labeling.

Serial dilutions (1:50, 1:100, 1:200, and 1:400) of test human serum were made using Tris-buffered saline (pH 7.0) and overlaid on prepared sections of rat ileum substrate. Because of the importance of phosphorylation affecting immunoreactivity with some antineuronal antibodies (16), phosphate-free Tris-buffered saline was used for all primary antibody dilutions. Polyclonal rabbit IgG anti–high-molecular-weight neurofilament (NF-H) antibody (17) (courtesy of Dr. Gerald Shaw, Department of Neurosciences, University of Florida) diluted 1:200 in Tris-buffered saline was used as a neuronal marker in double-labeling experiments. This antibody was produced by injecting rabbits with recombinant fusion protein NF-H and produces high titer neurofilament staining on rat brain substrate, specifically binding to purified NF-H subunits on immunoblots. Sections were incubated with samples of human and NF-H antisera as primary antibodies for double-labeled, indirect immunofluorescence microscopy as described previously (18). Goat $F(ab')_2$ specific for human Fc of IgG (Sigma Immunochemicals, St. Louis, MO), 1:40 dilution, linked to fluorescein isothiocyanate, and anti–rabbit whole molecule IgG linked to Texas red isothiocyanate (Sigma Immunochemicals), 1:20 dilution, were overlaid as the secondary detecting antibodies. Fluorescent detecting antibodies were crossed, and antineurofilament antibody and human serum were each omitted in early trials to exclude the possibility of nonspecific staining.

To absorb antinuclear antibodies from the sera, 500 μl of all serum samples positive for antimyenteric neuronal antibodies was rocked overnight at 37°C with 20 mg of lyophilized calf thymus extract (Sigma Immunochemicals), diluted in Tris-buffered saline to an effective dilution of 1:50. Unfortunately, there was not sufficient serum from patient 26 for calf thymus extract absorption. After centrifugation, the supernatant from the absorbed sera and from the unabsorbed sera were each diluted as before and overlaid on Hep-2 cells (ProTrac, Kerrville, TX) to demonstrate absence of residual ANA and, therefore, adequate absorption. Absorbed and unabsorbed sera were overlaid on cryostat sections of rat ileum, labeled with fluorescent-conjugated antibodies as before, and examined for fluorescence. Similar absorption studies were done with 10 positive scleroderma and 2 MCTD patient sera using rat whole brain homogenate, brought to an effective serum dilution of 1:60 using PBS/BSA, rocked overnight at 4°C, and centrifuged. The resultant supernatants at 1:60 dilution and unabsorbed serum at 1:60 dilution were overlaid on cryostat sections of rat ileum and Hep-2 cells using indirect immunofluorescence techniques and double-labeled with antineurofilament antibody as before.

Double-labeled specimens were viewed with an Axiophot immunofluorescence microscope (Zeiss, Oberkochen, Germany). Photographs were made, and qualitative evaluations of myenteric neuronal staining were made in a blinded fashion. Immunoreactivity for each dilution of serum was judged as positive (+) if the serum revealed strongly positive neuronal staining and negative (−) if staining was absent or equal to the background.

Cytoskeletal protein preparation. To determine if circulating antibodies reacted with cytoskeletal proteins, as has been reported in both connective tissue disease patients and normal individuals (19–21), we used intermediate filaments isolated from rat after the method of Shaw (22) and Shelanski (23) and actin prepared from chicken gizzard (Sigma Immunochemicals) and rat skeletal muscle (24).

Immunoblots. The intermediate filament preparation and actin protein were each transferred in 2× sample buffer with SDS to nitrocellulose after the method described by Towbin (25) using a Pharmacia Phastgel system (Uppsala, Sweden) (7.5% homogeneous gel, nonreducing conditions). Individual lanes were cut out of the nitrocellulose after appropriate blocking and incubated with mouse monoclonal antibodies for NF-H (NE14), medium-molecular-weight neurofilament (NN18), and glial fibrillary acidic protein (GA5), rabbit antiactin antibody (Sigma Immunochemicals), and six human sera positive for myenteric neuron staining (1:100–1:400 dilutions) as primary antibodies. Dilutions of 1:2,500 in 1% gelatin of peroxidase-conjugated goat $F(ab')_2$ anti–mouse, anti–human, or anti–rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used as secondary antibodies.

Homogenates of rat brain in 5 ml of 50 mM Mes and 2 mM EGTA and 1 mM $MgCl_2$ (pH 6.8) buffer were separated on 12.5% SDS-PAGE gels, and separated proteins were transferred to nitrocellulose membranes. Staining of separated proteins with known positive ANNA-1 were compared with results obtained using scleroderma sera with antimyenteric neuronal antibodies. Mouse anti–β-tubulin antibody (Sigma Immunochemicals) was used as a positive control.

Statistical analysis. Numerical data of groups were compared by Chi-square analysis with Yates correction (26). A P value > 0.05 is not significant.

Results

19 of the 41 patients with scleroderma had serum which stained myenteric plexus neurons using indirect immunofluorescence techniques (Table II). Two patterns were observed: homogeneous staining of neuron cell bodies but not nuclei or axons, and granular staining of neuron cell bodies and nuclei but not axons (Figs. 1 and 2). Of those sera which stained in a granular pattern, smooth muscle staining was also often seen. There was no difference in staining pattern when rat ileum was compared with lower esophagus, stomach, and colon. When positive serum was absorbed using lyophilized calf thymus extract, Hep-2 cell antinuclear staining was removed completely, but staining of myenteric neurons persisted in 15 out of 18 positive sera studied (Fig. 3). There was not enough serum available for further studies in 1 of the 19 positive patients (patient 26). In three sera from scleroderma patients, calf thymus extract absorption abolished myenteric neuronal immunoreactivity. When positive sera in 10 scleroderma patients were absorbed with rat whole brain homogenate, in all 10 the myenteric plexus staining and Hep-2 cell antinuclear staining were both diminished; in many instances, one absorption with brain homogenate eliminated both antinuclear and antineuronal staining (Fig. 4). The immunofluorescence pattern of staining seen with paraneoplastic sera was similar but clearly distinguishable as a different pattern (Fig. 5) from the staining demonstrated with the scleroderma sera (Figs. 1 and 2).

The presence of Raynaud's phenomenon in scleroderma patients was significantly associated with antimyenteric neuronal antibodies in their sera ($\chi^2 = 14.9$, $P < 0.001$). Of the scleroderma patients whose sera stained myenteric neurons, only one patient did not report Raynaud's phenomenon. Nucleolar ANA fluorescence patterns were found in 20% (3 out of 15) of patients positive for myenteric neuronal staining compared with 13% (3 out of 20) without ($\chi^2 = 1.3$, P = NS). Speckled ANA fluorescence patterns were found in 40% (6 out of 15) of positive

Table II. Serologic Characteristics of Scleroderma Patients

Patient	Myenteric neuron staining*	FANA Hep-2 cells	MOLT-4 Western blots‡	Double diffusion
1	–	1:640 centromere pattern	cenP-A, B, C, ?D, other	
2	+1:400 G	1:640 fibrillarin	(–)	
3	+ > 1:400 D	1:640 speckled pattern	30-, 46-, and 76-kD bands	
4§	+1:400 D	1:640 homogeneous with some nucleolar pattern	95- and 46-kD bands	Scl-70
5	+ > 1:400 D	1:640 diffuse homogeneous and nucleolar	Weak 37-kD bands	
6	–	1:160 homogeneous pattern	Weak 97-, weak 46-kD bands	
7	+1:100 G	1:2,560 centromere pattern	Doublet > 100, probable cenP-A	
8	–	1:160 diffuse fine speckled pattern	Weak 46-kD band	
9	–	1:640 centromere pattern	cenP-A	
10	+1:200 D	1:640 diffuse homogeneous, diffuse speckled	46-kD band	
11	–	n/a	n/a	
12	–	None detected	41-, 46-, weak 68-kD bands	
13	+ > 1:400 G	1:640 speckled pattern	Weak 18-, weak 23-, strong 31-, 42-, and 68-kD bands	RNP, SSA/Ro
14	–	1:640 centromere pattern	cenP-A, B, C	
15	+ > 1:400 G	1:640 centromere pattern	cenP-A, cenP-B, cenP-C	
16	–	1:640 centromere pattern	Weak cenP-A	
17	+ > 1:400 G	1:640 speckled pattern	68-, > 116-, 42-kD bands and (RNP)	RNP
18	–	1:640 centromere pattern	cenP-A, cenP-B, cenP-C	
19	+ > 1:400 G	1:640 homogeneous pattern	Strong 46–52-, 60-, weak 95-kD bands	SSA/Ro, SSB/La
20	–	1:40 homogeneous	(–)	
21	–	1:160 diffuse fine speckled pattern	Weak 69-, and 79-kD band	
22	–	1:640 centromere pattern	(–)	
23	–	1:640 discrete speckles	Strong 82-kD band	
24	–	1:640 centromere pattern	Weak cenP-A	
25	–	n/a	n/a	
26	+ > 1:400 G	1:640 centromere and homogeneous pattern, and nuclear membrane	cenP-A, weak cenP-B, weak cenP-C	
27	–	1:640 centromere pattern	cenP-A, B, C, others	
28	–	1:160 homogeneous pattern	(–)	
29	+1:200 D	1:640 fine speckled/cytoplastic pattern	46-, 52-kD bands	
30	–	1:40 diffuse fine speckled	78-kD band	
31§	+ > 1:400 G	1:640 fine speckles with occasional nucleolar pattern	(–)	
32	+ > 1:400 G	1:640 homogeneous and nucleolar pattern	92-, 78-, weak 46-kD bands	Scl-70
33	–	1:640 diffuse fine speckled pattern	> 116-, 100-, 95-, 46-, and 26-kD bands	Scl-70, other
34	–	1:640 granular nucleolar and homogeneous pattern	Two bands > 116-kD 116-kD, weak 68-kD bands	
35	+ > 1:400 G	1:640 homogeneous pattern	42-, 80-, Doublet > 100-kD bands	
36	+1:100 D	1:160 homogeneous, nucleolar	Weak 46-kD band	
37	–	1:640 centromere pattern	(–)	
38	–	1:640 centromere pattern	cenP-A	
39§	+1:50 G	1:640 homogeneous, scattered speckled pattern	Approximately 200-kD band	
40	+1:200 D	n/a	n/a	
41	+ > 1:400 G	1:640 speckled pattern	30-, 40-, 46-, strong 68-, weak > 100-kD band	RNP

* D, diffuse neuronal staining pattern; G, granular neuronal staining pattern; > 1:400 means endpoint titer exceeds 1:400; – means negative for myenteric staining; n/a means not available for further studies (patients expired or were lost to followup). ‡ cenP-A (17-kD band), cenP-B (94-kD band), cenP-C (> 100-kD band); § serum which subsequently was negative after thymus absorption.

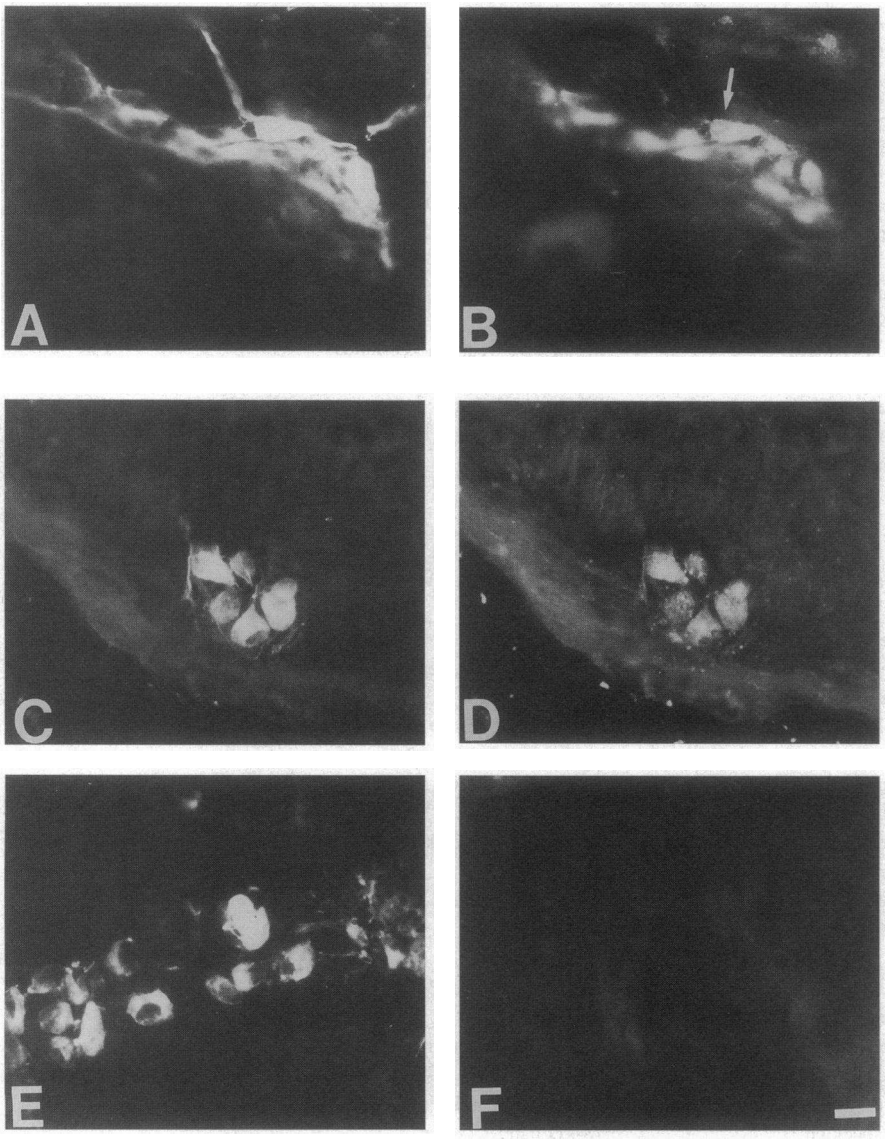

Figure 1. Representative double-labeled indirect immunofluorescence microscopy of rat ileum. *A* and *B* are corresponding double-labeled views of the same longitudinal section of ileum. *C* and *D* are corresponding views of another section of ileum, and *E* and *F* are corresponding views of a third section of ileum. In *A, C,* and *E,* the tissue was stained with the neurofilament antibody NF-H (1:200 dilution) to allow localization of many of the myenteric neuronal cell bodies and processes. In *B* (1:200 dilution, serum from patient 40) and *D* (1:400 dilution, serum from patient 29), scleroderma sera stained myenteric neuronal cell bodies diffusely, with more intense staining in the cytoplasm than in the nucleus (*B, arrow*). Axonal processes did not stain. In *F,* serum (1:100) from a control subject failed to stain neurons. Bar = 20 μm.

patients compared with 30% (7 out of 23) without myenteric staining ($\chi^2 = 1.78$, P = NS). The absence of anticentromere antibody and the presence of myenteric neuron staining were associated ($\chi^2 = 14.7$, $P < 0.001$), thus, only 3 out of 15 scleroderma patients with antimyenteric neuronal antibodies showed positive patterns for anticentromere staining.

Serum antibody profiles are summarized in Table II. The heterogeneity of antinuclear antibody reactivities among the positive scleroderma patients studied is shown in Fig. 6 (15). Antibodies reacting with myenteric neurons were found not only in diffuse, generalized scleroderma, but also among patients with limited scleroderma, CREST, morphea, and a few patients with overlap syndromes or undifferentiated connective tissue disorders. 11 out of 16 patients with antibodies reacting with myenteric neurons showed clinical or laboratory manifestations of GI dysfunction, however, 19 out of 25 patients without demonstrable antimyenteric neuron antibodies also showed GI dysfunction ($\chi^2 = 1.6$, P = NS).

None of the sera from 22 normals or 5 patients with idiopathic GI dysmotility stained myenteric neurons. In addition, none of the sera from 20 patients with clearcut rheumatoid arthritis showed positive staining. 6 sera from 20 SLE patients showed positive antimyenteric neuronal staining, but in each case absorption with thymus extract completely extinguished neuronal staining. 6 out of 10 MCTD patients' sera showed positive antimyenteric neuronal staining. After thymus extract serum absorption and whole brain homogenate absorption, two MCTD patients' sera still showed positive myenteric neuronal staining as well as persistent positive antinuclear staining with Hep-2 cells. Both of these patients showed extremely high ANA levels (1:5,120 and 1:20,480) and hypergammaglobulinemia.

Human serum which demonstrated positive immunoreactivity in myenteric neurons did not bind intermediate filaments or actin on Western blots. In parallel, polyclonal rabbit anti-actin, mouse monoclonal anti–NF-H, anti–medium-molecular-weight neurofilament protein, and anti–glial fibrillary acidic protein antibodies all showed strong positive Western blot staining on homologous substrates (data not shown).

Western blots of rat brain homogenates stained with positive scleroderma sera demonstrated weakly positive bands which clearly differed from the pattern of staining with paraneoplastic sera containing ANNA-1 antibody (data not shown).

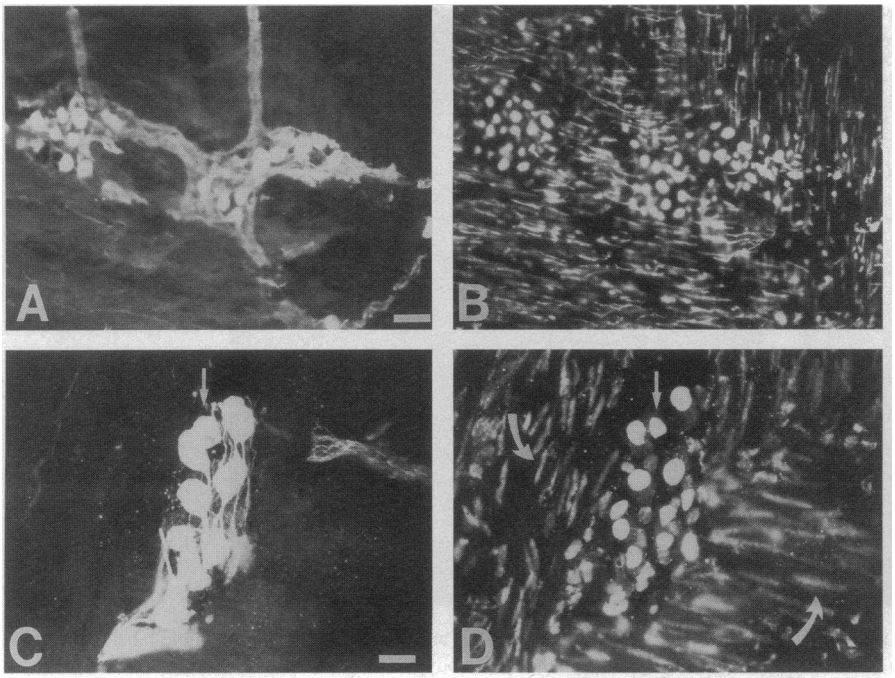

Figure 2. Representative double-labeled indirect immunofluorescence microscopy of rat ileum. *A* and *B* are corresponding double-labeled low power views (bar = 60 μm) of the same longitudinal section of rat ileum. *C* and *D* are corresponding double-labeled views of another section of ileum taken at higher power (bar = 20 μm). In *A* and *C*, the tissue was stained with the neurofilament antibody NF-H (1:200 dilution) demonstrating many of the myenteric neuronal cell bodies and processes. In *B* (1:200 dilution, patient 17) and *D* (1:200 dilution, patient 19), scleroderma sera intensely stained the area of the neuronal nucleus (*D, straight arrow*) with granular, less intense staining of the cytoplasm. Axonal processes do not stain. With this neuronal staining pattern, which differs from the staining in Fig. 1, smooth muscle cells (*D, curved arrows*) also demonstrated characteristic granular staining.

Discussion

Positive fluorescent ANAs are found in 95–97% (27, 28) of patients with scleroderma, but the presence of myenteric neuronal antibodies in scleroderma has not been described previously. In the present study, we report for the first time that some patients with scleroderma have myenteric neuronal antibodies in their sera. The myenteric neuronal antibodies which we have described appear to be relatively unique for scleroderma or scleroderma-like connective tissue disease patients because we did not find the antibody in any normals or patients with idiopathic GI dysmotility. Moreover, the antibodies were not found in any of 20 rheumatoid arthritis patients. Although a few of the 20 patients with SLE demonstrated positive neuronal staining, in each instance the immunoreactivity was eliminated by thymus preabsorption suggesting nonneuronal specific ANA. 2 of the 10 patients with MCTD showed the presence of antimyenteric antibodies but were associated with extremely high titer ANA and, as opposed to scleroderma sera, did not extinguish with brain extract absorption.

Scleroderma sera with the myenteric neuronal antibodies, when absorbed by calf thymus extract, lost ANA reactivity on Hep-2 cell substrate but maintained a myenteric neuronal staining pattern in 15 out of 18 instances; thus, the antibodies which react with myenteric plexus in our patients are not demonstrating a nonspecific ANA reactivity but are antineuronal in specificity. This was further substantiated by our demonstration that whole rat brain homogenate effectively absorbed antimyenteric neuronal antibodies from test sera. In this regard, the absolute

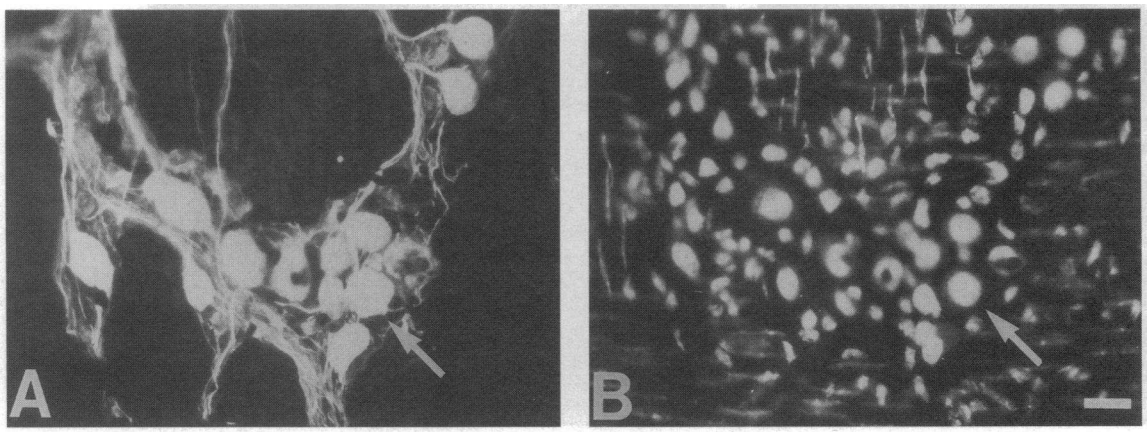

Figure 3. Representative double-labeled indirect immunofluorescence microscopy of rat ileum. *A* and *B* are corresponding double-labeled views of the same longitudinal section of ileum. In *A*, the tissue was stained with the neurofilament antibody NF-H (1:200 dilution) to allow localization of many of the myenteric neuronal cell bodies and processes. In *B*, scleroderma serum (patient 32) preabsorbed with calf thymus extract (effective dilution 1:40) to absorb antinuclear antibodies demonstrated no diminution in staining myenteric neurons (*arrow*) compared with unabsorbed serum. Bar = 20 μm.

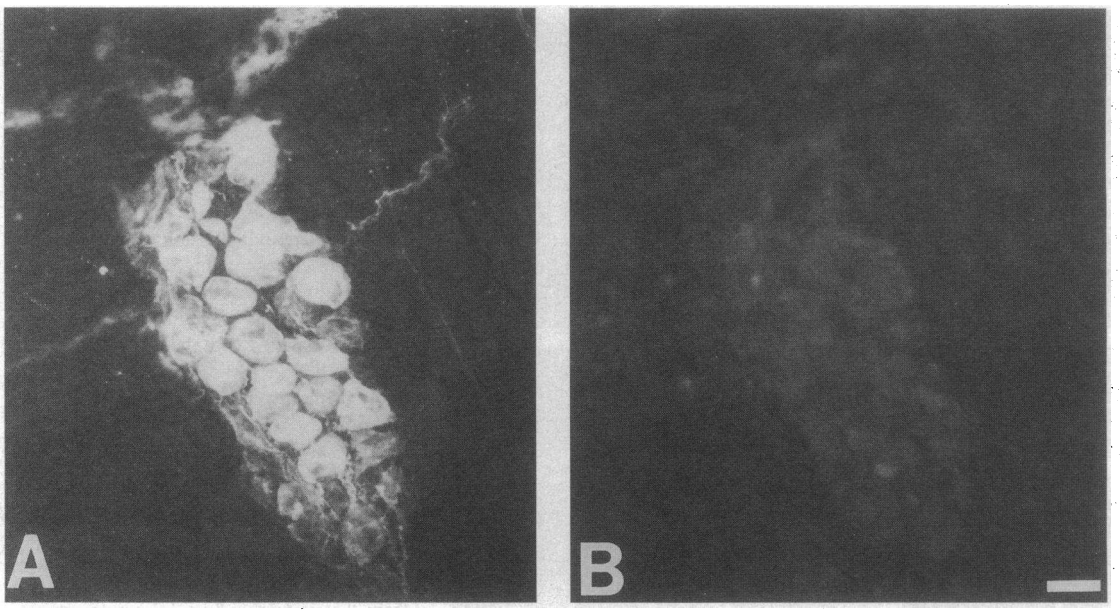

Figure 4. Representative double-labeled indirect immunofluorescence microscopy of rat ileum. *A* and *B* are corresponding double-labeled views of the same longitudinal section of ileum. In *A*, the tissue was stained with the neurofilament antibody NF-H (1:200 dilution) which demonstrated many of the myenteric neuronal cell bodies and processes. In *B*, scleroderma serum (patient 32), which showed positive myenteric neuronal staining, was preabsorbed with whole brain homogenate (effective 1:60 dilution) to absorb neuronal antibodies and demonstrated a dramatic absence of myenteric neuronal staining. Bar = 20 μm.

necessity for absorption of test sera with thymus extract to confirm specific neuronal staining was emphasized by our results, particularly with SLE sera where all apparent neuronal immunoreactivity was eliminated by thymus extract absorption. Anticytoskeletal antibodies in several immune-mediated diseases have been described but are relatively nonspecific, with circulating autoantibodies described in numerous disease states as well as in normals (19–21). The results of our immunoblots support that the antibodies we observed are not directed against intermediate filaments or actin. Moreover, as can be seen in Table II and Fig. 6, most of the patients' sera showed heterogeneous antinuclear antibody profiles often associated with scleroderma, such as anti–Scl-70 or characteristic bands on Western blots.

There was a negative reciprocal relationship of antimyenteric neuronal antibodies and anticentromere antibody. Thus, if antimyenteric neuronal antibodies were present, they were much less likely to be associated with the presence of anticentromere staining. Our results also demonstrated a significant number of patients with Raynaud's phenomenon having antimyenteric neuronal antibodies. This is of particular interest because of the association between esophageal dysmotility and Raynaud's

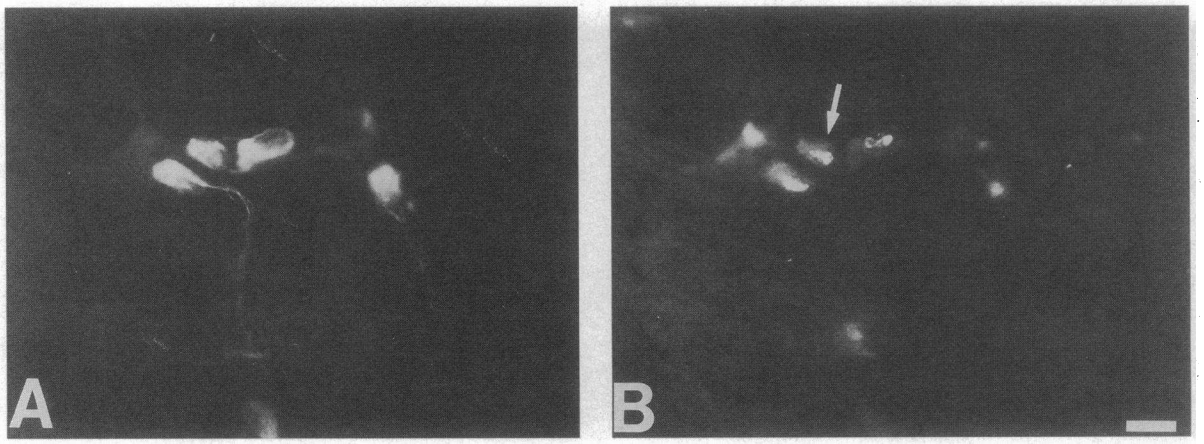

Figure 5. Representative double-labeled indirect immunofluorescence microscopy of rat ileum. *A* and *B* are corresponding double-labeled views of the same longitudinal section of ileum. In *A*, tissue was stained with the neurofilament antibody NF-H (1:200 dilution) which demonstrated many of the myenteric neuronal cell bodies and processes. In *B*, serum (1:400 dilution) from a patient with paraneoplastic GI dysmotility (courtesy of V. Lennon, Mayo Clinic) and known to contain ANNA-1 antibodies (10, 12) demonstrated intense clumped nuclear staining, with less cytoplasmic staining, and the absence of axonal staining. This pattern differed from that seen with scleroderma. Bar = 20 μm.

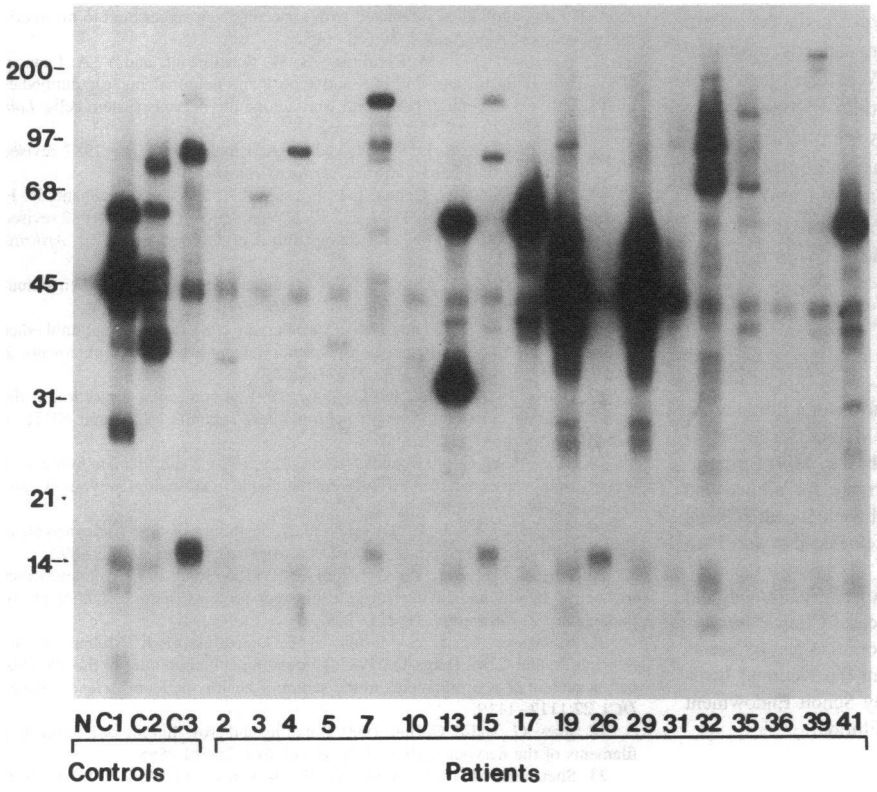

Figure 6. Immunoblotting results of patients with antimyenteric neuronal antibodies on MOLT-4 whole cell extracts. *N,* normal human serum; C1, control with antibodies to Sm, RNP, SSA/Ro, SSB/La; C2, control with antibodies to rRNP, Jo-1, Ku; C3, control with antibodies to SCL-70, centromere. Patient numbers refer to Table II. The autoradiograph was a long exposure to bring out weaker reactions with the result that anti-SSB/La in lanes *19* and *29* were overexposed.

phenomenon (4). Patients with Raynaud's phenomenon have shown prolonged esophageal rewarming compared with controls after cold challenge of the esophagus (29), suggesting that an ischemic process may be related. Our results demonstrate that a significant number of patients with Raynaud's phenomenon have antimyenteric neuronal antibodies. Gastrointestinal dysmotility may be the common link even though we were unable to demonstrate a significant association between GI dysmotility and presence of antimyenteric antibodies.

It has been suggested that scleroderma-associated GI dysmotility may begin primarily as a neuropathic process and later becomes a myopathic process (30). Cohen (7) showed that the lower esophageal smooth muscular response was intact in scleroderma after testing with methacholine (directly acting at the cholinergic receptor), edrophonium (enhancing acetylcholine effects), and gastrin I (proposed to increase the release of acetylcholine), suggesting a primary neurogenic defect. A later study by Cohen's group (8) of duodenal myoelectric activity in patients with scleroderma suggested that impaired intrinsic cholinergic pathways in scleroderma were implicated, but the mechanism for such neuropathogenicity is unknown. The provocative response of enhanced propagating small bowel motility with octreotide reported in scleroderma patients (31) indirectly supports the hypothesis that the process is neuropathic because such organized small bowel motor activity is mediated via the myenteric plexus.

The severity and clinical presentation of GI dysmotility in scleroderma is variable and may remain clinically occult. One problem in a disease like scleroderma is that patients often have major abnormalities in GI function which are subclinical and only apparent after invasive recording techniques are applied. Some patients with minimal or no apparent skin disease, the most prominent and easily recognized sign associated with scleroderma, have severe GI dysmotility, and others with severe skin disease may not have clinically apparent GI dysmotility until they have had the disease for several years. Examination of Tables I and II indicate that, in fact, six of our patients, though strongly positive for antimyenteric neuronal antibodies, denied GI symptoms and did not undergo extensive motility testing. 90% of patients with scleroderma undergoing esophageal manometry during some course of their illness will demonstrate esophageal aperistalsis in the lower two-thirds of the esophagus (the smooth muscle portion) or decreased lower esophageal sphincter pressure (32) with or without subjective dysphagia. Since clinical GI tract symptoms do not necessarily reflect GI dysmotility, our inability to link the presence of myenteric plexus neuronal antibodies in patients with such GI symptoms is not surprising. Clearly, a prospective study of GI tract motility in scleroderma patients will be required to determine whether myenteric antibodies predict early, neuropathic-based dysmotility.

Lennon et al. (10–12) have reported that some patients with cancer have circulating antimyenteric neuronal antibodies in which pathophysiologic changes in intestinal neuromuscular function are linked. These patients developed clinically evident dysmotility in histologically normal intestine. Similarly, early in the course of scleroderma, motility may be disturbed in the presence of normal esophageal smooth muscle (33). However, later on, at the extreme of disease, atrophy of smooth muscle, fibrosis of the submucosa and lamina propria, and thinning of the mucosa are seen. Even at that stage the myenteric plexus and skeletal muscle portions of the esophagus are pathologically normal at the light microscopic level (33, 34). The circulating antimyenteric neuronal antibodies which we are reporting in

scleroderma patients provide an attractive hypothesis to explain GI dysfunction on a neurogenic basis. The myenteric neuronal staining patterns, supported by preliminary Western blots, suggest a different neuronal antigen reactivity than recognized by ANNA-1 antibody (10–12). Our laboratories are pursuing studies to delineate the antigen(s) involved and the pathophysiologic significance of these observations in both animal and human studies in hope that this may shed some light on the poorly understood pathogenicity of scleroderma and provide a model for future therapy.

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