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Myasthenia Gravis-like Syndrome Induced by Expression of Interferon γ in the Neuromuscular Junction

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Summary

Abnormal humoral responses toward motor end plate constituents in muscle induce myasthenia gravis (MG). To study the etiology of this disease, and whether it could be induced by host defense molecules, we examined the consequences of interferon (IFN) γ production within the neuromuscular junction of transgenic mice. The transgenic mice exhibited gradually increasing muscular weakness, flaccid paralysis, and functional disruption of the neuromuscular junction that was reversed after administration of an inhibitor of acetylcholinesterase, features which are strikingly similar to human MG. Furthermore, histological examination revealed infiltration of mononuclear cells and autoantibody deposition at motor end plates. Immunoprecipitation analysis indicated that a previously unidentified 87-kD target antigen was recognized by sera from transgenic mice and also by sera from the majority of human MG patients studied. These results suggest that expression of IFN-γ at motor end plates provokes an autoimmune humoral response, similar to human MG, thus linking the expression of this factor with development of this disease.

Autoimmune responses to neuromuscular junction components in myasthenia gravis (MG),[1] such as the nicotinic acetylcholine receptor (nAChR), are largely pathogenic humoral responses. Patients suffer from progressive weakness of the voluntary muscles and many have autoantibodies against the nAChR, and a similar experimental condition, experimental autoimmune MG (EAMG), can be induced by the introduction of antibodies to the nAChR (1–3). Additionally, autoantibodies that impair neuromuscular transmission have been found in some MG patients who lack autoantibodies to nAChR (4–7).

Despite suggestions that viral or bacterial infections may trigger human MG, the molecular events leading to the development of the autoimmune response against the neuromuscular junction components remain unknown (8–10). The emphasis on primary pathogenic effects of autoantibodies is relatively uncommon among autoimmune phenomenon with the exception of an another neuronal disorder, stiff-man syndrome (11).

We sought to address the mechanism by which cells of the immune system become sensitized to components in the neuromuscular junction. Our previous studies have focused on the effects of cytokines elicited after infection or insult and that are capable of stimulating an immune response. While the autoimmune response in the neuromuscular junction of MG patients is quite distinct from that observed in other autoimmune disorders, we performed studies to determine whether molecules produced during the host response to infection could cause this type of disorder. We chose to study the effects of neuromuscular junction expression of the Th1 lymphokine IFN-γ, which has a multitude of immunostimulatory properties (12).

Materials and Methods

Production of ε-IFN-γ–transgenic Mice. The gene encoding IFN-γ was inserted into the BamHI restriction site of the 3.5-kb ε gene fragment containing the promoter region (13). An 8.8-kb XhoI/SpeI fragment containing the murine ε promoter, the murine IFN-γ

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[1] *Abbreviations used in this paper:* αBTX, α-bungarotoxin; EAMG, experimental autoimmune myasthenia gravis; EM, electron microscopy; EMG, electromyogram; H & E, hematoxylin and eosin; MG, myasthenia gravis; nAChR, nicotinic acetylcholine receptor; PSMP, postsynaptic membrane protein.

gene, and a terminator sequence were excised and purified for oo-cyte injection as described previously (14).

DNA Analysis. Southern blot analysis of genomic DNA was performed. 10 mg of genomic DNA, extracted from 2-cm-long tail segments, was digested with AccI enzyme overnight. The DNA was electrophoresed on 0.8% agarose gels and transferred to nitrocellulose membranes (NitroPure; Micron Separations Inc., Westboro, MA). Hybridization was performed with radiolabeled restriction fragments of the IFN-γ cDNA clone (Prime-It™ random primer labeling kit; Stratagene Inc., La Jolla, CA).

In Situ Hybridization. In situ hybridization confirmed that IFN-γ was expressed in neuromuscular junctions of ε-IFN-γ–transgenic mice. Skeletal muscles from 4-mo-old ε-IFN-γ–transgenic mice were placed in Bouin's fixative overnight. Deparaffinized sections were prehybridized for 2–3 h at 42°C in a buffer of 50% formamide, 0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, and 10 mM dithiothreitol. Hybridization with ^{35}S-labeled sense and antisense RNA probes (750,000 cpm/section) was done for 16 h at 42°C in a humidified chamber. This was followed by washing, dehydration, and drying. Sections were covered with nuclear track emulsion (NTB-2; Eastman Kodak Co., Rochester, NY), exposed for 1 wk, and developed. RNA probes were prepared by in vitro transcription of linearized plasmid containing IFN-γ cDNA. No significant signals were seen with sense probes.

Antibody Purification. The isolation and purification of ε-transgenic BALB/c mice and human IgG was performed by use of a protein A/G IgG purification kit (Pierce Chemical Co., Rockford, IL).

Serum Collection. Sera from MG patients and patients with non-myasthenic disorders were supplied by the laboratories of Drs. H. Fox and J. Lindstrom with the help of Dr. Marjorie Seybold (The Scripps Research Institute). The sera were either stored at 4°C or –70°C.

Anti-nAChR Antibody Assay. nAChRs were isolated from the mouse cell line BC3H-1 (15). The assay for anti-nAChR antibody was performed as follows: 5-μl aliquots of mouse sera were incubated overnight at 4°C with 10^{-9} M nAChR labeled with 2×10^{-9} M ^{125}I-αBTX in 100 μl of PBS (pH 7.5) containing 0.5% Triton X-100, and 10 mM NaN₃. 40 μl of 10% fixed *Staphylococcus aureus* A (Zysorbin; Zymed Labs, Inc., South San Francisco, CA) were then added and incubated for 75 min. After dilution with 1 ml of the same buffer, the *S. aureus*-antibody-labeled nAChR precipitate was pelleted for 5 min in a microfuge. The pellet was washed twice, and then the tubes were counted for radioactivity. A blank value obtained by using 5 μl of normal mouse serum in the assay was subtracted from all measurements. All assays were done in triplicate.

Protein Analysis. Protein analyses were performed on muscle homogenates, crude cell extracts, and purified nAChR preparations by use of SDS-PAGE. The muscle homogenate was prepared by disrupting muscle tissue in PBS with a homogenizer (Tissumizer; Tekmar, Cincinnati, OH), and the crude and purified nAChRs were prepared as mentioned above. All samples received an equal volume of 2× SDS-gel loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, electrophoresis grade, 0.2% bromophenol blue; 20% glycerol) and were boiled for 10 min. For homogenates, the samples were centrifuged at 14,000 rpm for 10 min, and the supernatants were transferred to fresh tubes. To perform gel electrophoresis, a 30-μg aliquot of protein muscle homogenates or an 8-μl aliquot of crude cell extracts (14 nM/μl) was loaded onto 1.5-mm-thick Tris-glycine 10% polyacrylamide slab gels (Tris-glycine electrophoresis buffer: 25 mM Tris base, 250 mM glycine,

pH 8.3, 0.1% SDS). After SDS-PAGE (16), proteins were electro-transferred for 1.5–2 h to membranes (Immobilon; Millipore, Bedford, MA) (transfer buffer: 39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). The membranes were subsequently probed with either purified IgG from the sera of ε-transgenic mice (1:100), sera from patients with MG, sera from patients with non-myasthenic disorders, or the rabbit anti-87-kD postsynaptic membrane protein polypeptide antibodies of #625 and #638 (1:2,000; gifts from Dr. R. L. Huganir, Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, MD). After being probed with IgG or sera, the bound antibodies were detected with peroxidase-conjugated goat anti–mouse IgG (1:5,000; Boehringer Mannheim Corp., Indianapolis, IN), goat anti–human Igs (1:1,000; Sigma Chemical Co., St. Louis, MO), or goat anti–rabbit IgG (1:2,000; Tago, Inc., Burlingame, CA). Finally, peroxidases on the membranes were visualized with enhanced chemoluminescence (Amersham Life Science, Amersham, UK).

Immunoprecipitations. To reveal the antigenic components reactive to transgenic mouse IgG, we performed immunoprecipitation using IgG from BALB/c transgenic mice, MG patients, and anti-87-kD–postsynaptic membrane protein polypeptide antibodies #625 and #638. The crude cell extracts were isolated from both mouse BC3H-1 and human TE671 muscle cell lines (17) that were precleared with protein A–Sepharose and protein G–Sepharose 4B Fast Flow beads (Sigma Chemical Co.). To form immunocomplex, the IgG preparations (100 μg for mouse IgG, 150 μg for MG patients, and 1 μg of anti-87-kD–postsynaptic membrane protein polypeptide antibodies #625 and #638) were added separately to 25 μl each of precleared nAChRs. After 1-h incubation at 4°C, the mixtures were reacted with excessive protein A and protein G–Sepharose beads. The antigen–antibody complexes bound to the beads were recovered by sedimentation by microfuge and washed with Tris buffer (0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.01 M EDTA, 2% Triton X-100), repelleted, resuspended in 30 μl of 1 × SDS loading buffer, heated in boiling water for 5 min, and pelleted in a microfuge. The proteins in both Sepharose bead–bound pellets and supernatants were analyzed by SDS-PAGE.

Histological and Immunocytochemical Analysis. Tissues were fixed in Bouin's fixative and processed for paraffin embedding. Sections were stained with hematoxylin and eosin (H & E) for histological examination and in situ hybridization experiments. Immunolabelings were performed on unfixed frozen sections. The entire labeling procedure was carried out at room temperature. All washes and reagents were in PBS. The nonspecific binding sites were blocked in 10% normal goat serum for 30 min. All antibodies were incubated 30 min and washed three times for 3 min each between the two steps. The primary antibodies were rat anti–mouse CD4 (PharMingen, San Diego, CA) and rat anti–mouse Mac-1 (Boehringer Mannheim Corp.). The secondary antibody was biotinylated rabbit anti–rat IgG, mouse adsorbed (Vector Labs, Inc., Burlingame, CA). Sections were incubated with an avidin–biotin peroxidase complex (ABC kit; Vector Labs, Inc.) and visualized with diaminobenzidine. Sections were counterstained with Papanicolaou stain "EA-65" or hematoxylin, dehydrated, and mounted. For double-immunofluorescence staining, the sequential staining technique was used. The nAChRs were stained with rhodamine-conjugated α-bungarotoxin (αBTX) (Molecular Probes, Inc., Eugene, OR) overnight at 4°C. The next day, they were continually stained with fluorescein-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) overnight at 4°C. All rinses and reagents were in PBS.

Electron Microscopy. For electron microscopy (EM) observations, mice were perfused with warmed 2.5% glutaraldehyde. Muscle was

removed and fixed in 2.5% glutaraldehyde, postfixed in 1% O_sO_4, dehydrated in graded ethanol, and embedded in araldyte. 0.5 to 1.0-μm sections were cut and stained with uranyl acetate and lead citrate before viewing with an electron microscope (model 101; Siemens, Karlsruhe, Germany) operating at 80 kV.

Electromyograms. Mice were anesthetized with chloral hydrate (100 mg/kg) and placed on a heating pad to allow maintenance of body temperature at 38°C by use of a heating lamp, temperature regulator, and thermistor probe. A stimulating needle electrode was placed at the Achilles tendon, and recording needle electrodes were inserted between the second and third and third and fourth digits of the left hind paw. A single supramaximal stimulus (4–6 V, 0.05 ms) was applied adjacent to the peroneal nerve, and the resulting electromyogram was recorded on an analogue storage oscilloscope. This was followed by a 1-s train of stimuli (the "initial" stimulus; 4–6 V, 0.05 ms, 5 Hz), after which the amplitude of each of the five electromyogram (EMG) recordings was measured from the screen and the difference between the amplitude of the first and fifth EMG recordings calculated as the percentage change. The system was then provoked by Faradization (4–6 V, 0.05 millisecond, 50 Hz) for 5 s before application of another 1-s train of stimuli (the "provoked" stimulus; 4–6 V, 0.05 millisecond 5 Hz). A decline of $\geqslant$10% in the amplitude between the first and fifth EMG recordings of either the initial or provoked stimuli was considered evidence of neuromuscular dysfunction (18). All animals were coded, and the investigator was unaware of the identity of individual mice.

Results

Derivation of ε-IFN-γ–transgenic Mice. To produce transgenic mice expressing IFN-γ in the postsynaptic membrane of the neuromuscular junction, we constructed a recombinant DNA plasmid that fused the regulatory sequences of the murine nAChR ε gene (13) to the coding sequences of the murine IFN-γ gene (Fig. 1). We chose to use the ε subunit of the nAChR because it is the last subunit transcribed during ontogeny, starting 1 d after birth (19). Expression of the IFN-γ gene from the ε subunit promoter would not, therefore, be expected to interfere with the development of neuromuscular junctions in embryos. Of 36 mice born, 10 proved to be transgenic when analyzed by Southern blot hybridization. Transgenic lines were maintained by breeding with BALB/c mice. Four transgenic lines were characterized extensively. Animals in one of these four lines weighed less than normal but had no apparent impairment of mobility at 5 mo of age. However, after the sixth month (late onset), muscular weakness became visible. The remaining three lines had similarly re-
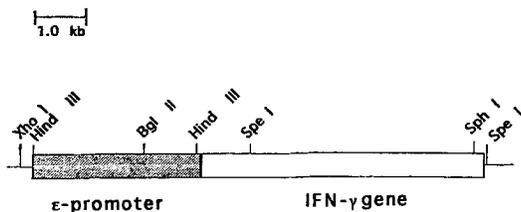
duced body weights but showed locomotor impairment by 6 wk of age, indicating the early onset of muscular weakness. In situ hybridization demonstrated the presence of IFN-γ RNA in individual dispersed cells within the muscles of transgenic mice. The observed expression pattern was consistent with perijunctional localization of the lymphokine in mice from all transgenic lines (Fig. 2).

Characterization of Appearance and Behavior in ε-IFN-γ-transgenic Mice. At birth, the transgenic mice appeared indistinguishable from their nontransgenic littermates. However, after weaning, their weight gain slowed, resulting in an ~25% lower weight for 2-mo-old early-onset transgenic mice compared with their nontransgenic littermates. In late-onset mice, the weight differences were not as noticeable at this stage. The mature transgenic animals then lost 5–10% of their body weight each month during the observation period of 7 mo. Their fur was ruffled, and their distinctive posture featured a humped back and drooping head and tail, indicating muscle weakness. Further evidence of muscular dysfunction was their slowed motion, difficulty in climbing cage sides, and long periods of inactivity. Enforced exercise accentuated their motor impairments. Similar motor dysfunction was reported for EAMG induced by the injection of nAChR (1, 20–25).

Anticholinesterase Treatment. Administration of the anticholinesterase drug neostigmine temporarily eliminated signs of muscle weakness, as is the case in MG. Within 15 min after the transgenic mice were given neostigmine (0.015 mg/kg body weight intraperitoneally), they began to traverse the cage actively and groom themselves. This restoration of motor activity by neostigmine suggests that an increase in the concentration of acetylcholine, as a result of acetylcholinesterase inhibition, augmented the efficiency of neurotransmission. Thus, this muscular weakness seems to be a defect of neurotransmission rather than the contractile properties of the muscle cells.
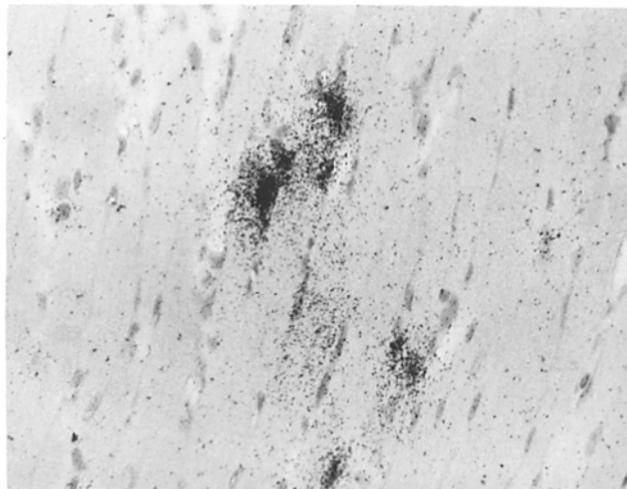
Figure 2. In situ hybridization confirming that IFN-γ was expressed in neuromuscular junctions of ε-IFN-γ-transgenic mice. Skeletal muscles from 4-mo-old ε-IFN-γ-transgenic mouse. ×160.

Figure 1. Structure of recombinant ε-IFN-γ gene. The gene encoding IFN-γ was inserted into the BamHI restriction site of the 3.5-Kb ε gene fragment containing the promoter region (13). An 8.8-kb XhoI/SpeI fragment containing the ε promoter, the IFN-γ gene, and a terminator sequence were excised and purified for oocyte injection as described previously (14).

Electrophysiological Investigation. To determine whether the morphological changes were associated with functional disturbances, electrophysiologic testing was performed by use of a modification of the procedure described by Pachner and Kantor (18). None of nine control nontransgenic mice exhibited a change in EMG amplitude of ≥10% during tetanic (5 Hz) stimulation, either before or after provocation (Fig. 3 *A*). No spontaneous EMG activity was noted in any transgenic mouse, and normal biphasic EMGs were recorded after stimulation. Of the early-onset transgenic mice, four out of five exhibited decreases of ≥10% in EMG amplitude during the initial stimulation and also after provocation (Fig. 3 *B*). Decreases of ≥10% were also noted in three out of six 6-mo-old late-onset transgenic mice during the initial stimulation and in five out of six after provocation (Fig. 3 *C*). Neostigmine treatment attenuated the decline in EMG amplitude after provocation in a late-onset transgenic mouse that had previously shown a marked decline (Fig. 3 *D*). The mean changes in EMG amplitude during tetanic stimulation for each group before and after provocation are shown in Fig. 3 *E*. These decreases in electrophysiologic test scores indicate a disturbance in neuromuscular transmission that closely resembles that noted in MG patients (26) and EAMG subjects (18).

Histopathology of Muscle in ε-IFN-γ–transgenic Mice. By light microscopy, H & E-stained sections of muscles showed infiltration by lymphocytes, polymorphonuclear, and mononuclear cells (Fig. 4 *A*). Individual myofibrils showed degenerative changes, including the appearance of target fibers. Leukocyte cell subtypes were identified as MHC class II–expressing macrophages and occasional CD4$^+$ T cells (Fig. 4, *B* and *C*). Both CD4$^+$ T cells and macrophages are present in human MG and have been implicated in the generation of autoimmune responses to nAChR (27). In addition, numerous plasma cells were identified by anti-IgG antibody in the adjacent lymph node. In other organs, including the thymus, no apparent abnormality was found.

Ultrastructural Analysis. EM of skeletal muscles from both early- and late-onset transgenic mice revealed ablation of junctional folds of the sarcolemma (Fig. 5), suggesting a reduction in the number of nAChR (28, 29). Strikingly, macrophages were consistently present adjacent to the morphologically al-
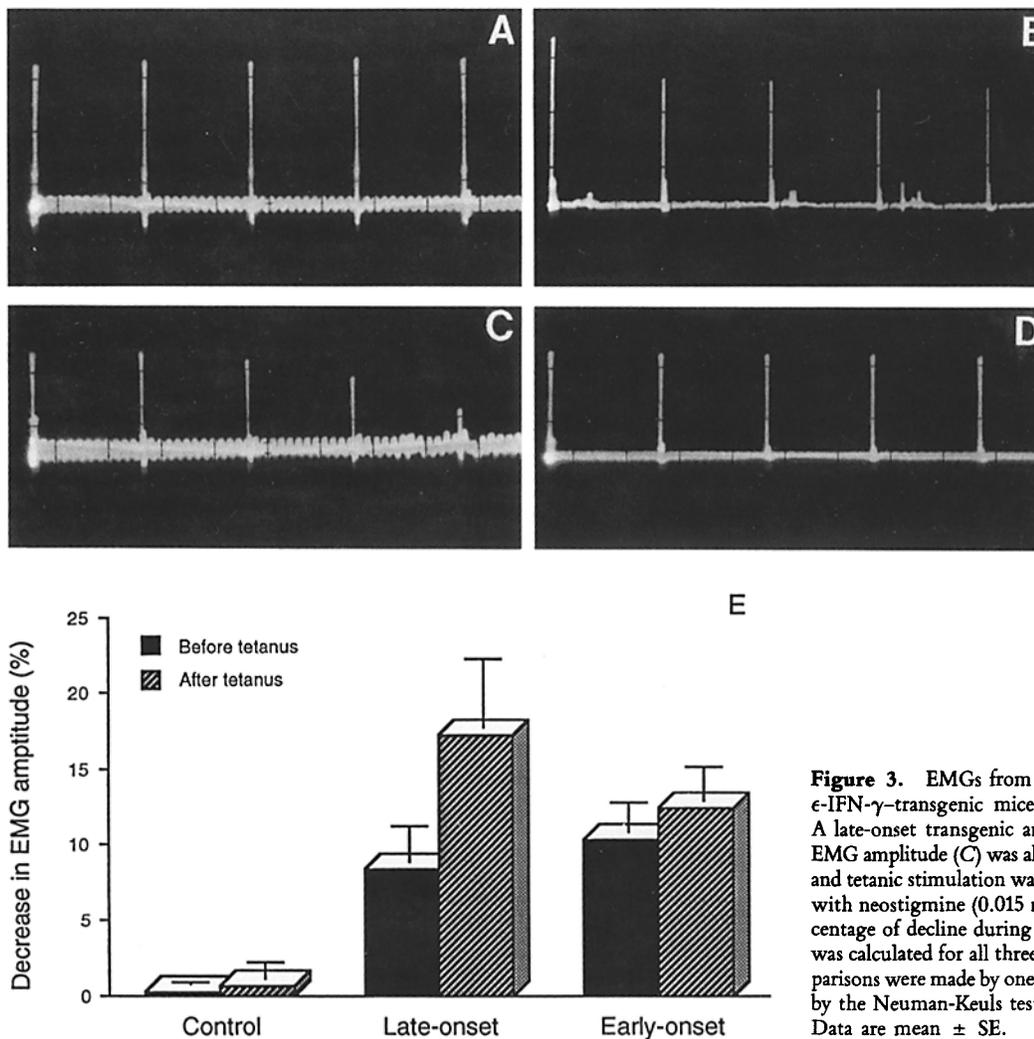

Figure 3. EMGs from control (*A*) and early-onset (*B*) ε-IFN-γ–transgenic mice after tetanic stimulation (30). A late-onset transgenic animal with a marked decline in EMG amplitude (*C*) was allowed to recover from anesthesia, and tetanic stimulation was repeated 80 min after treatment with neostigmine (0.015 mg/kg, i.p.) (*D*). The mean percentage of decline during the initial and provoked stimuli was calculated for all three groups (*E*), and statistical comparisons were made by one-way analysis of variance followed by the Neuman-Keuls test when the *F* test gave $p < 0.05$. Data are mean ± SE.
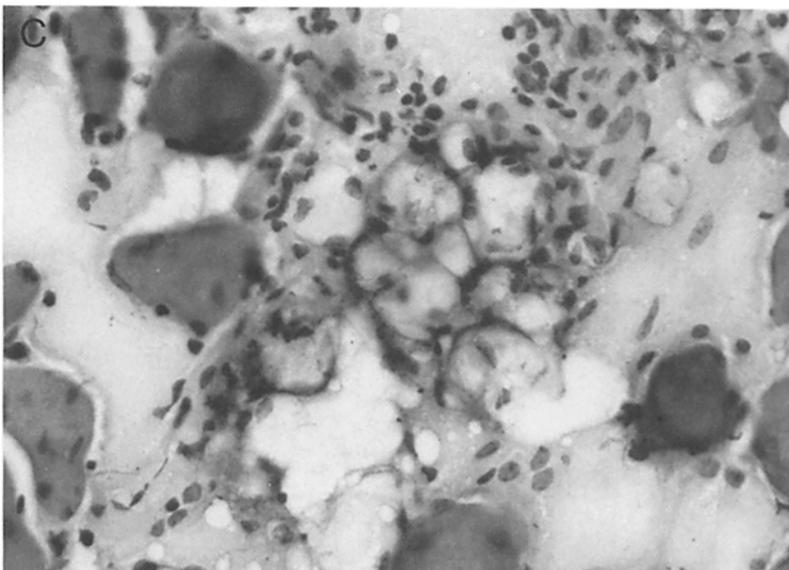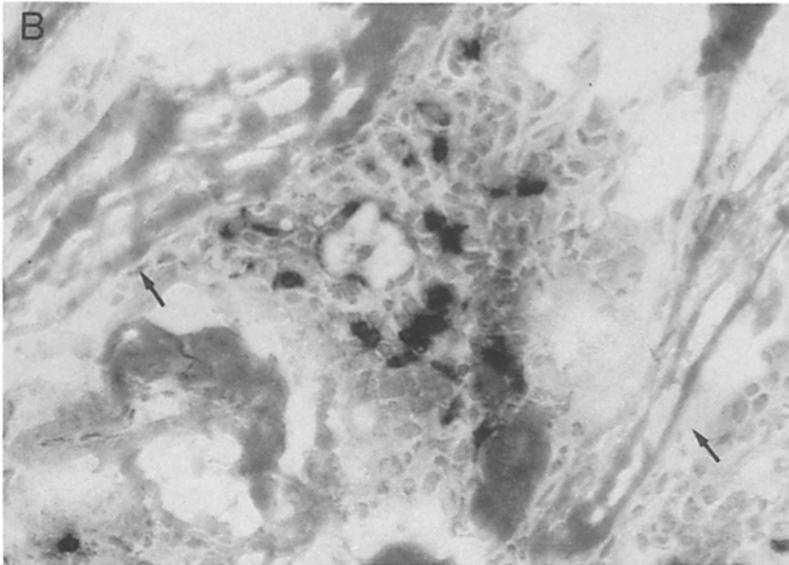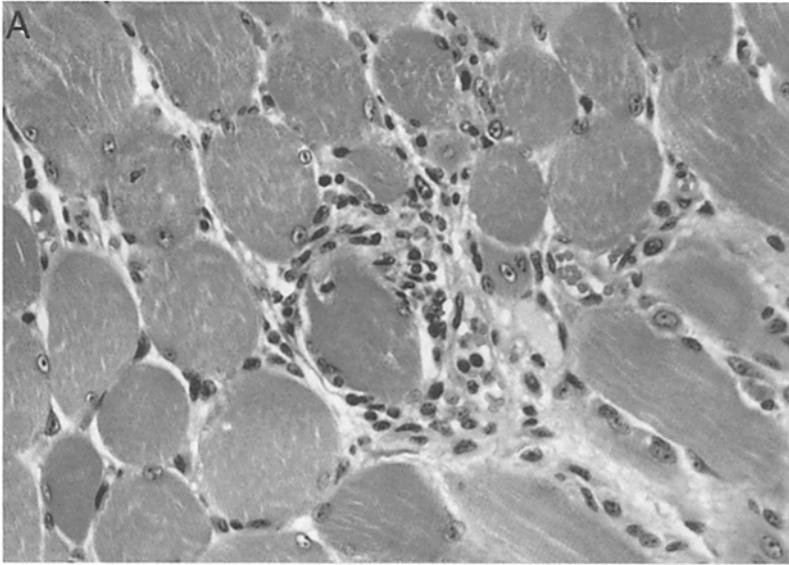
Figure 4. Cellular infiltrates on skeletal muscles from 4-mo-old ε-IFN-γ mice. (A) Limb muscle showing monocytic infiltration (H & E stain). (B) CD4+ T cells (center) and atrophic muscle fibers (arrows) shown by immunoperoxidase labeling. (C) Mac-1+ cells. For B and C, cryostat sections were labeled with rat anti-CD4 or rat anti-Mac 1 antibodies. The secondary antibody was biotinylated rabbit anti–rat IgG. Sections were incubated in avidin–biotin peroxidase complex and visualized with diaminobenzidine. Sections were counterstained with Papanicolaou stain "EA-65" or hematoxylin, dehydrated, and mounted. ×160.
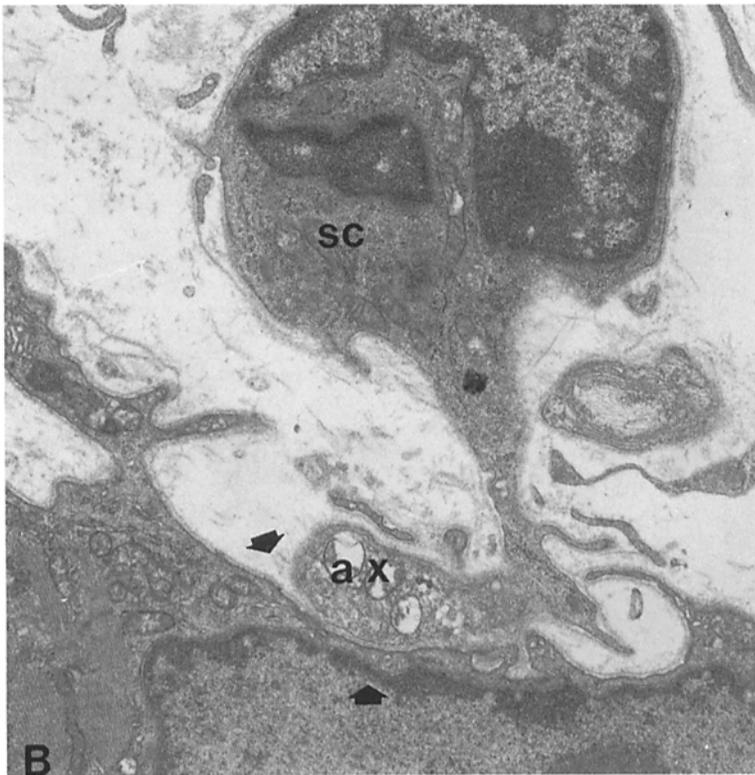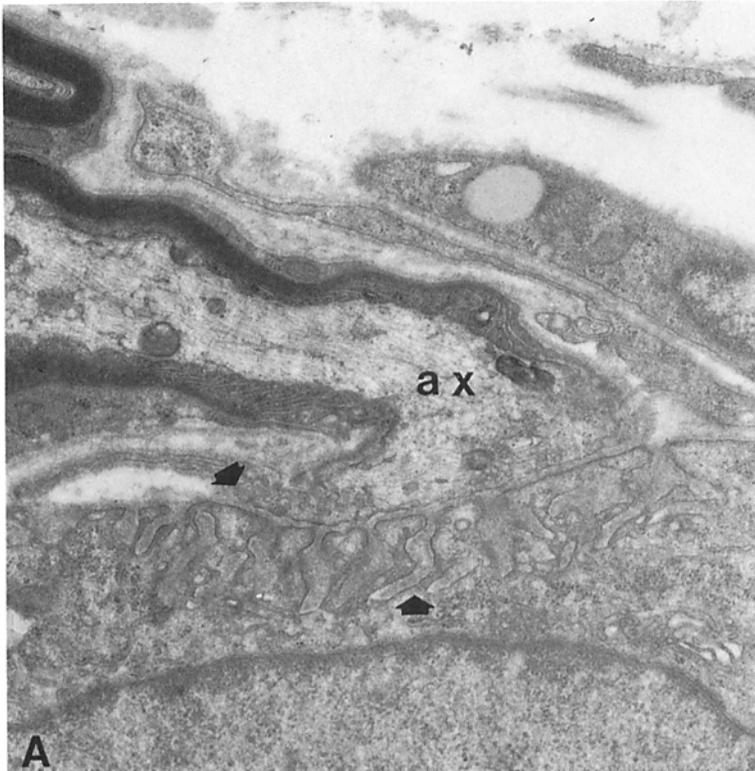
Figure 5. Comparison of motor end plates in the intercostal muscles of control (*A*) and ε-IFN-γ-transgenic (*B*) mice (×21,000 and ×16,000, respectively). The control mouse has a myelinated axon (*ax*) terminating at the neuromuscular junction with its complex junctional infoldings (*arrows*). The ε-IFN-γ-transgenic mouse lacks these junctional infoldings (*arrows*) and displays axonal (*ax*) shrinkage as well as retraction of the Schwann cells (*sc*) from the terminal axon.

tered neuromuscular junctions (Fig. 6). Simplification of the postsynaptic membrane occurs commonly in the neuromuscular junctions of MG patients (30–32) or animals with chronic EAMG (28), but phagocytic invasion does not. In fact, phago-cytic invasion of neuromuscular junctions is usually noted only in acute or passively transferred EAMG. Furthermore, in the adjacent lymph nodes, the appearance of plasma cells containing ultrastructural characteristics of IgG hypersecre-
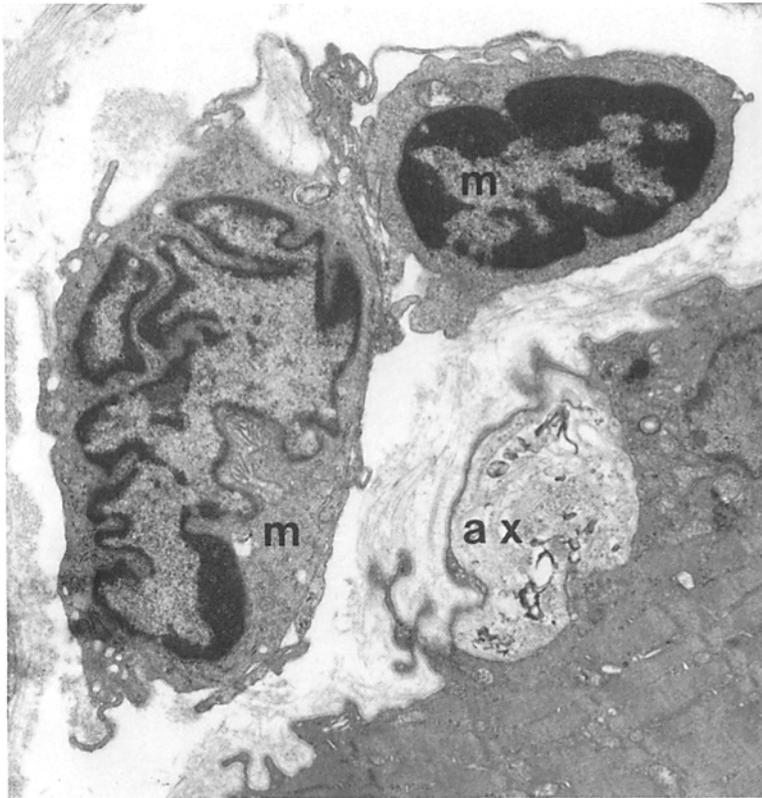
Figure 6. Intercostal muscle from an ε-IFN-γ–transgenic mouse. Mononuclear cells (*m*) were regularly noted adjacent to abnormal terminal regions of axons (*ax*). ×12,300.

tion (Russell bodies) indicated the activation of the humoral immune system.

Autoantibody Deposition. Immunohistochemical staining experiments were performed with FITC-conjugated antibody against mouse IgG on cryostat sections of muscle from transgenic and control mice. Intense staining by mouse IgG was visible as deposits believed to be at the motor end plates of skeletal muscles (Fig. 7 B). In 2-mo-old mice of the early-onset group, staining was restricted to a few weakly staining motor end plate structures. However, by 4 mo of age, intense staining of a large number of motor end plates was observed. This progression from a few weakly stained end plates to many strongly stained ones during the interval between 2 and 4 mo of age correlated with the time period during which clinical symptoms arose. The late-onset mice showed a similar staining pattern only after they reached ⩾6 mo of age. The localization of IgG deposition to the motor end plate region was confirmed by double-labeling experiments using rhodamine-conjugated αBTX to probe for nAChRs. Indeed, the fluorescein label colocalized with the αBTX labeled (Fig. 7, A and B). Our observations of the αBTX-labeled muscle tissues revealed that most synaptic clefts from transgenic mice appeared as slender elongated structures, a shape that was quite distinct from those of nontransgenic mice. Furthermore, the few fairly normal appearing synaptic clefts of these mice were not double labeled with the fluoresceinated antibody. The IgG deposits in synaptic clefts of the transgenic mice accumulated in circular foci, some of which also stained with αBTX. Such round foci were never detected

in tissues from nontransgenic mice tested with the αBTX label. Studies in which these areas were double stained with hematoxylin indicated that the round foci did not correspond to nuclei, and possibly represent capping structures of membrane-bound antibody. The purified IgG from ε-transgenic mouse sera also positively stained the motor end plates of BALB/c muscles (Fig. 7, C and D). No staining was observed in BALB/c muscle when purified IgG from BALB/c sera was used (data not shown). These results strongly suggest that the IFN-γ expressed in the neuromuscular junction induced a humoral autoimmune response toward components in the motor end plates.

Target Antigen Detection. A large percentage of MG patients exhibit humoral autoimmunity to motor end plate constituents, most commonly directed at the nAChR. To investigate whether our IFN-γ–transgenic mice had a similar immune response, we looked directly for anti-nAChR autoantibodies by use of labeled αBTX and performed Western blots on purified IgG from transgenic and control mice. As our source of antigen, we used the mouse cell line BC3H-1, which expresses muscle nAChRs and is presumably derived from a motor end plate (33). We failed to detect any autoantibodies to nAChRs in the serum from transgenic mice (data not shown). Interestingly, however, we were able to consistently detect an 87-kD band on Western blots of BC3H-1 extracts that appeared with serum from all 12 transgenic mice analyzed. This band did not appear when brain extracts were used as antigen (data not shown).

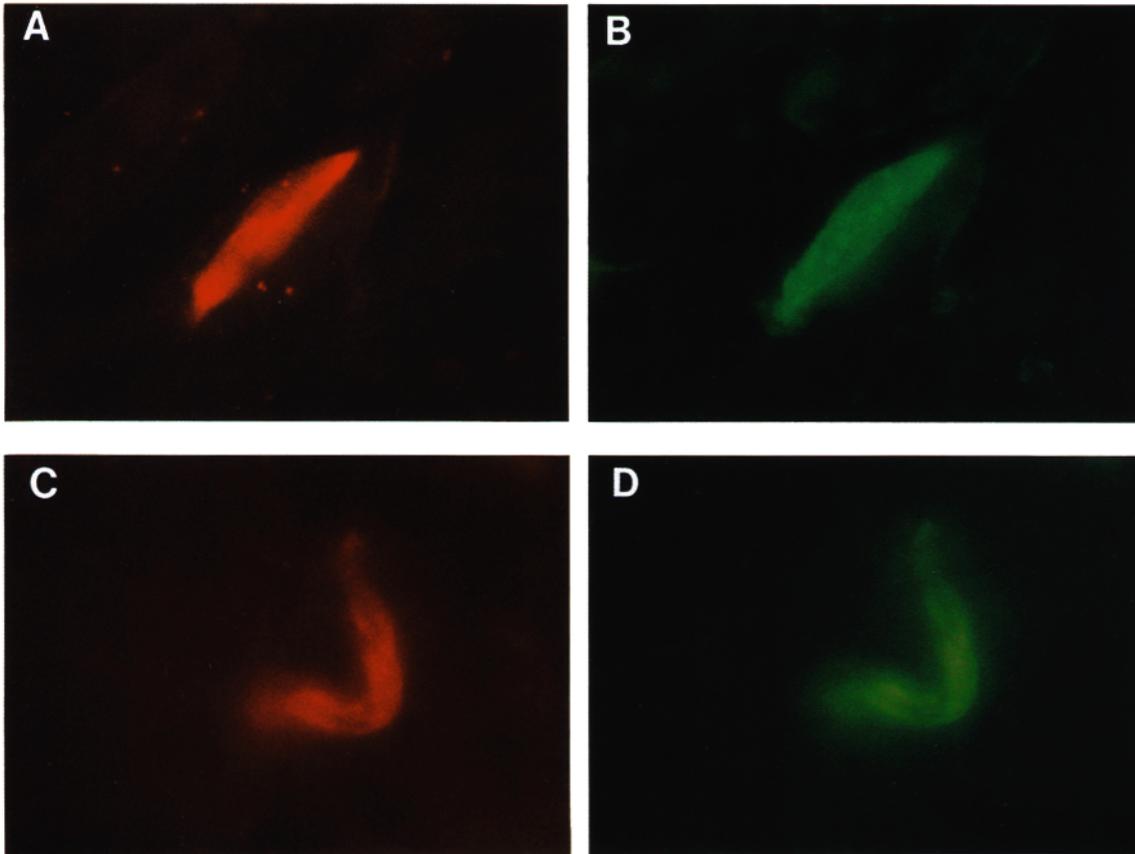Since a category of MG patients are "seronegative," having

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Figure 7. Detection of IgG deposition in transgenic motor end plates and the binding of transgenic IgG to BALB/c motor end plates (mouse age: 4 mo). The motor end plate was first identified by rhodamine-conjugated αBTX. (*A*) Muscle from transgenic mouse; (*c*) muscle from BALB/c mouse. (*B*) The presence of IgG in the same motor end plate of a transgenic mouse was visualized by FITC-conjugated goat anti–mouse IgG. (*D*) BALB/c muscle was incubated first with transgenic IgG followed by FITC-conjugated goat anti–mouse IgG; the motor end plate was also labeled, indicating that transgenic mice contained antibodies to some component(s) of motor end plates. When IgG from nontransgenic control mice was used to stain BALB/c muscles, no staining was observed (not shown). *A*, *B*, ×450; *C*, *D*, ×1,000.

no measurable anti-nAChR antibodies, we wondered whether the 87-kD protein found in the transgenic mice represented an additional target antigen that was shared with seronegative MG patients. We therefore obtained sera from seronegative MG patients and performed similar Western blot anal-
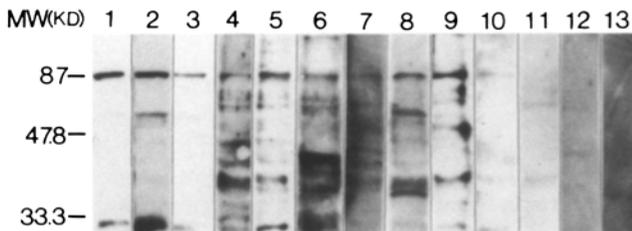

Figure 8. Reactivity of transgenic mice and human MG patients to components in the BC3H-1 cell line extract. Western blotting analysis revealed that antibodies from individual mice and MG patients consistently recognized an 87-kD protein in the extracts. (Lanes *1–3*) IgG from transgenic mice. (Lanes *4–7*) Sera from MG-seronegative patients. (Lanes *8–10*) Sera from MG-seropositive patients. (Lanes *11–13*) sera from patients with non-MG autoimmune disorders. Note the absence of a 87-kD band.

ysis. Strikingly, these samples contained an 87-kD protein, which was labeled by sera from 15/15 seronegative MG patients. The protein recognized by mouse purified IgG and human sera appeared identical in size. We also detected immunoreactivity to the 87-kD band in 8/14 seropositive MG patients. Importantly, the 87-kD protein was not recognized by purified IgG from either BALB/c mice or sera from 7/8 of nonmyasthenic patients (Fig. 8 and Table 1). To determine whether the 87-kD protein was similar in mice and humans, we performed cross-immunoprecipitation experiments. We immunoprecipitated extracts from the murine muscle cell line BCH3-1 and human tumor cell line TE671 using purified transgenic mouse IgG or human MG IgG, and Western blots were performed on the immunoprecipitated material with sera from the other species. We were able to detect the human immunoprecipitation product with the mouse IgG, and vice versa (Fig. 9).

An 87-kD postsynaptic membrane protein has been previously reported in vertebrate muscle (34, 35). To determine whether this 87-kD protein was the one recognized by IgG from transgenic mice (ε-IgG), we obtained anti-87-kD-postsynaptic membrane protein antibodies (anti-87-kD-PSMP-

Table 1. *Results of Western Blot Analysis in Mice and Seronegative and Seropositive MG Patients*

Source of IgG or Sera	Positive 87-kD protein % Positive/Total	
ε-IFN-γ-transgenic mice	100	(12/12)
BALB/c mice	0	(0/6)
Seronegative MG patients	100	(15/15)
Seropositive MG patients	57.2	(8/14)
Nonmyasthenic disorder	14	(1/8)

Crude cell extract was prepared from the BC3H-1 cell line. There was an 87-kD protein labeled with purified IgG from 12 transgenic mice or sera from 15 seronegative and 8 seropositive MG patients. This 87-kD protein stained negatively with purified IgG from either BALB/c mice or sera from most of the nonmyasthenic autoimmune disorder patients. One patient out of eight showed positive staining.

Ab, a generous gift from R. Huganir) and prepared immunoprecipitates from our crude extracts. The antigen supernatants were collected and allowed to reprecipitate with either anti-87-kD-PSMP-Ab or ε-IgG. SDS-PAGE showed that anti-87-kD-PSMP-Ab and ε-IgG each quantitatively removed its own respective protein, but failed to recognize the other (data not shown). These results demonstrate that there was no cross-reactivity between anti-87-kD-PSMP-Ab and ε-IgG and strongly suggest that our crude extract 87-kD protein is not the same as postsynaptic membrane 87-kD protein in the motor end plates.

Discussion

While the perturbations used to produce transgenic mice cannot, by definition, copy naturally occurring conditions, we have demonstrated some striking similarities between the ε-IFN-γ-transgenic mice and aspects of human MG. These include overt muscular weakness, simplification of the postsynaptic membrane, progressively declining EMG responses to stimulation, restoration of motor activity by anticholinesterase drugs, and infiltration of muscles by cells of the immune system. Additionally, both human MG and our transgenic mice demonstrate deposition of antibody at motor end
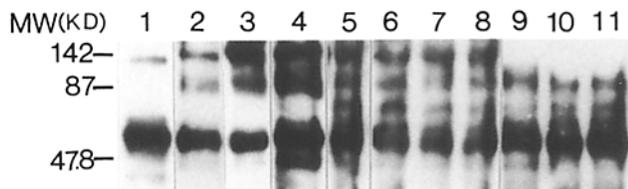

Figure 9. The proteins immunoprecipitated from BCH3-1 extracts by use of transgenic mouse IgG or MG patient IgG. (Lane *1*) Control BALB/c IgG. (Lanes *2–4*) Probed with individual transgenic mouse IgG. (Lanes *5–11*) Probed with individual human MG patient IgG.

plates, which in our mice occurs during the preclinical period. Lastly, we have demonstrated the presence of antibody directed against an 87-kD protein in crude mouse BC3H-1 and human TE671 extracts that is shared by transgenic mice, human seronegative MG patients, and the majority of seropositive MG patient sera we studied.

The pathogenic significance of immunological sensitization to the 87-kD antigen remains to be determined. Preliminary immunofluorescence studies using sera from transgenic mice on cultured BC3H-1 cells demonstrated cell surface localization of the 87-kD antigen. It is possible that these antibodies may bind to epitopes of a protein closely associated with the nAChR and are capable of modulating its function indirectly. The possible physical association between the 87-kD antigen and the nAChR is supported by the fact that we could detect the 87-kD band with ε-IFN-γ sera on Western blots of partially purified nAChR. This suggests, but does not prove, that the 87-kD protein could coprecipitate with the nAChR. Previous studies have shown that Ig obtained from "seronegative" patients, when injected into experimental animals, caused loss of nAChRs and decreased amplitudes of motor end plate potentials, thus providing evidence for the presence of antibody directed against some postsynaptic component of the neuromuscular junction (36, 37). Our data on humans and mice suggest that this antigen could be the 87-kD band we have identified. Further experimentation is warranted to address this point.

The histological lesions in this model consisted of inflammatory cell infiltrates that could result from the response to viral infection or autoimmune disease. These inflammatory infiltrates, composed mainly of macrophages, were quite different in cellular composition from the more diverse pancreatic infiltrates (including CD8⁺ T and B cells) previously observed in transgenic mice expressing IFN-γ in the islets of Langerhans (38). Interestingly, pancreatic expression of quite disparate signals such as the Th2 cytokine IL-10 are capable of recruiting infiltrates that are surprisingly similar in cellular composition to the pancreatic infiltrates observed with IFN-γ expression (39). Thus, identical signals (IFN-γ) lead to pronounced differences in inflammatory responses in different tissues, while disparate signals lead to similar recruitment patterns in the same tissue. This might be mediated at the level of the tissue endothelium, which has been demonstrated in vivo to respond to localized expression of IFN-γ (40).

The expression of IFN-γ leads to pathogenic responses mediated by distinct arms of the immune system in each tissue. In the neuromuscular disease of our transgenic mice, the autoantibody deposits at the motor end plates imply autoimmunity in the humoral compartment. However, no CD8 cells could be identified within the transgenic muscles, implying the lack of participation of a cell-mediated response. This is quite distinct from transgenic mice expressing IFN-γ in the pancreas, where no humoral response could be identified by either islet cell antibodies or by studying the humoral response to specific islet constituents (41). In contrast, pancreatic expression of IFN-γ leads to infiltration by CD8 cells and the development of cell-mediated immunity to islet constituents

(41, 42). This Th1 lymphokine, which is associated with stimulating such cell-mediated responses, in the milieu of the neuromuscular junction induces humoral autoimmunity. This leads us to speculate that tissue-specific tropic factors could contribute to a localized immune response, enhancing, for example, a humoral rather than a cell-mediated response (such as is seen in human MG). Such factors might normally participate in a host's defense of a specific region. Indeed, access to "sterically" sequestered areas/antigens, such as the neuromuscular junction, would necessitate emphasis on humoral immunity over a cell-mediated response. This effect could account for the disparity in the primarily humoral response to glutamic acid decarboxylase antigen in the brain, which leads to the neurological disease stiff-man syndrome, whereas a cell-mediated response to the same tissue-specific antigen in pancreatic islets is associated with the development of insulin-dependent diabetes mellitus (43, 44).

The IFN-γ–transgenic model is useful because of the inheritable nature of trangenes, providing a mouse strain for prolonged experiments. In contrast, some EAMG animals exhibit a self-limiting disease that subsides several months after primary immunization. Several differences exist between our transgenic model and human MG. As in EAMG (45), our transgenic mice had no evidence of hyperplasia or thy-moma in any of seven thymuses examined, whereas ∼50% of MG patients develop hyperplasia, and ∼10% also develop benign or malignant tumors. Additionally, although we routinely saw macrophages in the vicinity of disrupted neuromuscular junctions, and the postsynaptic membrane displayed the simplification characteristic of both EAMG and human MG, the overall amount of inflammatory cell recruitment in our transgenic model is much higher than that reported for MG. This is probably due to more avid recruitment as a secondary phenomenon to endothelial cell activation, which is regulated by the constitutive expression of IFN-γ.

In transgenic mice, we have demonstrated a humoral response to motor end plate components that results from localized lymphokine expression. Our results suggest that human MG could be initiated by immune defense molecules expressed in response to an environmental insult such as infection. Sensitization to the nAChR or other motor end plate components might then occur as a secondary event. Inflammation and autoantibodies in the neuromuscular junction can substantially impair neuromuscular transmission and cause clinically apparent muscular weakness. Although the entire pathway from cytokine to disease is not totally clear, our work directly links such inflammatory mediators to MG.

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