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Gastrointestinal hypomotility with loss of enteric nicotinic acetylcholine receptors: active immunization model in mice

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Abstract

Background—Autoimmune gastrointestinal dysmotility (AGID) is a limited form of dysautonomia. The only proven effector to date is IgG specific for ganglionic nicotinic-acetylcholine receptors containing $\alpha 3$ subunits ($\alpha 3^*$ -nAChR). Rabbits immunized with recombinant $\alpha 3$ -polypeptide produce $\alpha 3^*$ -nAChR autoantibodies, and profound AGID ensues. Human and rabbit $\alpha 3^*$ -nAChR-specific-IgGs induce transient hypomotility when injected into mice. Here we describe success and problems encountered inducing gastrointestinal hypomotility in mice by active immunization.

Methods—We repeatedly injected young adult mice of seven different strains susceptible to autoimmunity (spontaneous diabetes or neural antigen immunization-induced myasthenia gravis or encephalomyelitis) with: i) $\alpha 3$ -polypeptide, intradermally, or ii) live $\alpha 3^*$ -nAChR-expressing xenogeneic cells, intraperitoneally. We measured serum $\alpha 3^*$ -nAChR-IgG twice monthly, and terminally assessed blue dye gastrointestinal transit, total small intestinal $\alpha 3^*$ -nAChR content (radiochemically) and myenteric plexus neuron numbers (immunohistochemically, ileal-jejunal whole-mount preparations).

Key Results—Standard cutaneous inoculation with $\alpha 3$ -polypeptide was minimally immunogenic, regardless of dose. Intraperitoneally-injected live cells were potently immunogenic. Self-reactive $\alpha 3^*$ -nAChR-IgG was induced only by rodent immunogen; small intestinal transit

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AUTHOR CONTRIBUTIONS

JWM: Study concept and design, acquisition of data, analysis and interpretation of data, drafting of manuscript.

KEH: Acquisition of data, analysis and interpretation of data.

JPF: Study design and technical support.

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SJG: Study design and technical support.

YX: Material support and critical revision of the manuscript.

VAL: Study concept and design, analysis and interpretation of data, drafting of manuscript, critical revision of the manuscript for important intellectual content, obtained funding and study supervision.

DISCLOSURES

The authors disclose no conflicts. Serological testing for neural autoantibodies is offered on a service basis by Mayo Collaborative Service, Inc., an agency of Mayo Foundation. Neither Dr. Lennon nor her laboratory benefit financially from this testing.

slowing and enteric $\alpha 3^*$ -nAChR loss required high serum levels. Ganglionic neurons were not lost.

Conclusions & Inferences—AGID is inducible in mice by active immunization.

Accompanying enteric $\alpha 3^*$ -nAChR reduction without neuronal death is consistent with an IgG-mediated rather than T cell-mediated pathogenesis, as is improvement of symptoms in patients receiving antibody-depleting therapies.

Keywords

autoimmune dysmotility; dysautonomia; ganglionopathy; slow transit

Autoimmune gastrointestinal dysmotility (AGID) is a limited dysautonomia, occurring idiopathically and in paraneoplastic context.¹⁻³ Onset is typically subacute, with early satiety, abdominal pain, nausea, vomiting, weight loss and slow transit. Autoimmune serology aids the diagnosis. Autoantibodies specific for intracellular antigens are surrogate markers of peptide-specific T cell-mediated effector mechanisms. Anti-neuronal nuclear-type 1 autoantibody (ANNA-1/anti-Hu) and collapsin response-mediator protein antibody ([CRMP]-5) both predict underlying lung carcinoma or thymoma.^{1, 4} Autoantibodies specific for extracellular domains of plasma membrane antigens have pathogenic potential^{2, 3, 5}. Symptoms improve with antibody-depleting therapies (plasma exchange, B lymphocyte-depleting-IgG or high dose intravenous immune globulin).⁵⁻⁷ Specificities include ganglionic-type ($\alpha 3^*$) and muscle-type ($\alpha 1^*$) nicotinic acetylcholine receptor (nAChR) and neuronal voltage-gated calcium channel (N-type) or potassium channel-complex.^{5, 8} Most frequently identified is $\alpha 3^*$ -nAChR-IgG, the only autoantibody presently proven to cause AGID.^{5, 9} Rabbits immunized with human $\alpha 3$ -polypeptide (extracellular residues 1-205) readily produce autoantibody, and AGID signs parallel serum $\alpha 3^*$ -nAChR-IgG levels.¹⁰ Immune serum IgG (rabbit or human) transfers transient gastrointestinal hypomotility to mice.⁹ We now report AGID induction by actively immunizing mice.

MATERIALS AND METHODS

Cell lines and $\alpha 3$ -nAChR fusion protein

Human neuroblastoma cells IMR-32 (American Type Culture Collection [ATCC]) and SHSY-5Y (Sloan Kettering) were maintained in Gibco minimal essential medium with 10% FBS and 1% nonessential amino acids and Eagle MEM/Ham F12 medium (1:1, supplemented with 15% FBS). Human embryonic kidney cells (HEK-293, ATCC) were maintained in Dulbecco MEM (Gibco) with 10% bovine calf-serum. The stably-transfected HEK-293 line KX $\alpha 3\beta 4R2$ was derived and maintained as described.¹¹ Recombinant human $\alpha 3$ -polypeptide (residues 1-205 fused to GST) was produced from cDNA derived from IMR-32¹⁰.

Native $\alpha 3^*$ -nAChR preparation

Post-nuclear membrane fractions (isolated by differential centrifugation of homogenized IMR-32 cells grown subcutaneously in athymic nude mice and from cultured KX $\alpha 3\beta 4R2$ cells) were extracted in buffer (10 mM Na₃PO₄, 1 mM EDTA, 100 mM NaCl, 0.02% NaN₃, pH 7.4) containing 2% Triton X-100. Supernates were clarified centrifugally (100,000 g, 30 minutes).

$\alpha 3^*$ -nAChR-IgG

Mouse serum (5 μ L duplicate specimens; serial 10-fold dilutions as necessary) was mixed with solubilized $\alpha 3^*$ -nAChR (50 fmol complexed with 10-fold excess 125 I-epibatidine). Antibodies were detected by radioimmunoprecipitation.^{10, 12, 13}

Animal procedures

The Mayo Institutional Animal Care and Use Committee approved the experiments. Mouse strains susceptible to autoimmunity (spontaneous diabetes mellitus,¹⁴ or neural antigen-induced myasthenia gravis¹⁵ or encephalomyelitis¹⁶) were purchased from Jackson Laboratories (Bar Harbor, ME), acclimated 10 days in a conventional facility, anesthetized with 4% isoflurane/4% O₂ and injected with fusion protein emulsified in complete Freund's adjuvant (*i.d.*, six sites;), and with killed *B. pertussis* organisms (20 billion, *s.c.*, four sites). Live cells were injected *i.p.*, 10 million in 0.25 mL PBS, initially with killed *B. pertussis* organisms, *s.c.*, as adjuvant. Mice were weighed weekly and bled alternate weekly.

Small intestinal transit

Brilliant Blue FCF (100 μ L) was gavaged via 18-gauge needle. Mice were killed 30 min later (CO₂ asphyxiation); small intestine was removed (gastroduodenal to ileocecal junction). Transit (distance of dye front from gastroduodenal junction) was expressed as percent total small intestinal length: 100% when dye reached ileocecal junction; 0% when dye remained in stomach.

Neuronal density quantification

Myenteric plexus neurons (ANNA-1-immunoreactive perikarya)¹⁷ were enumerated in coded photomicrographs of muscularis propria whole-mount preparations (1 cm of ileo-jejunal junction tissue; **Fig. 1B**). Images from four corners and center of each (five randomly-picked fields of 0.59 mm²) were captured by Olympus AX70 fluorescence microscope, 20X objective. Automated object counting (SvCell; Svision LLC, Bellevue WA, employing proprietary soft matching algorithms to segment ANNA-1 positive somata) was validated on a randomly chosen set of 143 images. Numbers obtained from manual counts correlated significantly with numbers obtained by the SvCell program (**Fig. 1C**). Significance between $\alpha 3$ -nAChR-immune and control immune groups was compared by *t*-test.

Small intestinal $\alpha 3^*$ -nAChR extraction

After luminal flushing with Kreb's solution (mM: 137.4 Na⁺, 5.9 K⁺, 2.5 Ca²⁺, 1.2 Mg²⁺, 134 Cl⁻, 15.5 HCO₃⁻, 1.2 HPO₄²⁻, and 11.5 glucose, saturated with 97% O₂ and 3% CO₂), tissue was weighed and nAChR was extracted as above.

$\alpha 3^*$ -nAChR quantification

Cell and tissue extracts were incubated with 125 I-epibatidine, with and without non-radioactive epibatidine in 10-fold excess, and quantified using glass fiber filtration.¹⁸

RESULTS

Despite repeated intradermal injections of recombinant human $\alpha 3$ -polypeptide 1-205 and adjuvants, serum autoantibody detection was infrequent (Table), and levels lower than 3.00 nmol/L (predicts severe AGID in immunized rabbits).¹⁰ No gastrointestinal dysmotility signs were evident.

Live cell immunization

We next investigated the immunogenicity of SHSY-5Y neuroblastoma cells expressing endogenous $\alpha 3^*$ -nAChR. Three weeks after injection, $\alpha 3^*$ -nAChR-IgG was detected in 50% of C57BL/6J mice; 100% were seropositive after re-inoculating on day 28. Levels peaked after a third inoculation (day 120), far exceeding serum levels of mice injected with $\alpha 3$ -polypeptide (Table). Mice receiving human kidney cells (HEK-293) intraperitoneally were seronegative. Despite high $\alpha 3^*$ -nAChR-IgG levels, no mouse had signs of AGID. Small intestinal transit ($80 \pm 11\%$) was not significantly different from control values ($67 \pm 12\%$; Fig. 2A). The discontinuous dye distribution pattern in the small intestine of both groups indicated normal peristalsis.

Next, we investigated the immunogenicity of KX $\alpha 3\beta 4R2$ cells (stably expressing rat $\alpha 3$ and $\beta 4$ nAChR subunits, and 5-fold more $\alpha 3^*$ -nAChR protein than neuroblastoma cells; Fig. 2B). After three intraperitoneal inoculations, 29 of 32 mice (91%) were $\alpha 3^*$ -nAChR-IgG-seropositive; levels were lower than in mice receiving neuroblastoma cells (Table). However, terminal tissue harvest revealed pooling of gavage dye in the proximal intestine of 14 mice (45%), suggesting loss of coordinated peristalsis (Fig. 1A). Furthermore, despite lower frequency and levels of $\alpha 3^*$ -nAChR-IgG, small intestinal transit was significantly slowed ($49 \pm 16\%$; Fig. 2A).

Severe hypomotility despite relatively modest $\alpha 3^*$ -nAChR-IgG levels raised the possibility that antibody induced by rodent $\alpha 3^*$ -nAChR immunization was more reactive with rodent antigen than with human. Specific testing revealed serum IgG reactive with rodent $\alpha 3^*$ -nAChR in 100% of mice immunized with rat $\alpha 3^*$ -nAChR-expressing cells, but in no mouse immunized with human $\alpha 3^*$ -nAChR-expressing cells (Table). Rat and mouse $\alpha 3$ -nAChR subunit amino acid sequences are identical, but human $\alpha 3$ -nAChR subunit differs in 10 of 205 extracellular domain residues accessible to circulating IgG. These data implicate autoantibody as the cause of slowed small intestinal transit and loss of coordinated peristalsis.

To evaluate histometric evidence of neuronal cytotoxicity, we counted neurons in whole-mount preparations of jejuno-ileal junction tissue (Fig. 1B). Neuron numbers did not differ significantly in enteric ganglia of mice immunized with human $\alpha 3^*$ -nAChR, rodent $\alpha 3^*$ -nAChR or non-transfected HEK-293 cells (Fig. 2C; respectively, 100 ± 19 neurons/mm², 102 ± 14 neurons/mm² and 113 ± 16 neurons/mm²). Thus, neuronal cytotoxicity did not explain the hypomotility.

Total small intestinal $\alpha 3^*$ -nAChR content (γ -emission from Triton-X100-solubilized membrane macromolecules ligated with ¹²⁵I-epibatidine and captured on glass fiber filters) was 85% less in tissues of mice immunized with rodent $\alpha 3^*$ -nAChR-expressing cells than in tissues of mice immunized with human $\alpha 3^*$ -nAChR-expressing-cells or with non-transfected cells (respectively, 61 ± 41 fmol/g, 409 ± 173 fmol/g and 384 ± 91 fmol/g; Fig. 2D).

DISCUSSION

“Self”- $\alpha 3^*$ -nAChR-reactive autoantibody production is required to develop AGID in actively immunized mice. Profound intestinal $\alpha 3^*$ -nAChR loss without loss of enteric neurons indicates that hypomotility was not attributable to neuronal death. The most plausible mechanism is $\alpha 3^*$ -nAChR internalization and degradation following its cross-linking in neuronal plasma membranes by circulating IgG. This mechanism underlies $\alpha 1^*$ -nAChR loss from muscle cells exposed to serum from rodents with experimental myasthenia gravis¹⁹ and partial aquaporin-4 water channel loss from astrocytes exposed to serum from

patients with neuromyelitis optica.²⁰ $\alpha 3^*$ -nAChR internalization is compatible with: i) reduction of agonist-induced depolarization of $\alpha 3^*$ -nAChR-expressing cells following application of human autoantibody,²¹ ii) transient gastrointestinal hypomotility in mice injected with $\alpha 3^*$ -nAChR-IgG⁹ and iii) rapid patient recovery from AGID symptoms following antibody-depleting therapies.^{7, 22} Reproducible induction of a mouse model of AGID by active immunization will enable detailed analysis of genetic susceptibility determinants, pathogenic and immunoregulatory mechanisms, and implementation of novel preclinical therapeutic trials.

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Figure 1.

Quantification of small intestinal transit and enteric neurons in mice. **(A)** Pooling of blue dye in the proximal small intestine of a mouse seropositive for rodent $\alpha 3^*$ -nAChR-IgG (upper image) contrasts with the segmented distribution in a control mouse immunized with HEK293 cells (lower image). Arrows indicate gavaged dye front. **(B)** Representative immunohistochemical image of myenteric neuronal cells in whole mount jejunum-ileal junction tissue of a mouse seropositive for rodent $\alpha 3^*$ -nAChR-IgG. **(C)** Correlation of neuron numbers obtained by SvCell automated counting with neuron numbers obtained by hand counting from a data set of 143 images ($R^2 = 0.85$, Slope of trend line fitted to data = 1.01).

Figure 2.

Intestinal transit, myenteric neuronal counts and small intestinal $\alpha 3^*$ -nAChR content of mice immunized with live cells. (A) Small intestinal transit in mice immunized with cells expressing rodent $\alpha 3^*$ -nAChR is significantly slower than in mice immunized with human $\alpha 3^*$ -nAChR-expressing cells or with control cells. (B) $\alpha 3^*$ -nAChR protein in lysates of cell lines used as immunogens was quantified by glass fiber filtration assay in terms of macromolecules specifically binding ^{125}I -epibatidine. (C) Myenteric neuron numbers per mm^2 of intestinal wall, calculated from images as illustrated in Fig. 1B. (D) Intestinal $\alpha 3^*$ -nAChR protein (fmol/g) in mice immunized with cells expressing rodent $\alpha 3^*$ -nAChR was significantly less than in mice immunized with cells expressing human $\alpha 3^*$ -nAChR or cells lacking $\alpha 3^*$ -nAChR.

Table

Antibody responses of mice immunized with α 3-nAChR polypeptides or human cells expressing endogenous or recombinant α 3*-nAChR

Mouse strain	Immunogen [†]	Serum α 3*-nAChR-IgG values (nmol/L)					
		Human			Rodent		
		n	Frequency (%)	Median (range)	Frequency (%)	Median (range)	
C57BL/6J	Human α 3, residues 1 – 205	6	3 (50)	0.07 (0.05 – 0.12)	2 (33)	0.07 (0.04; 0.10)	
AKR	Human α 3, residues 1 – 205	7	0	–	0	–	
SJL	Human α 3, residues 1 – 205	12	1 (8)	0.05	3 (25)	0.07 (0.05-0.10)	
129S/J	Human α 3, residues 1 – 205	8	0	–	nt	nt	
NOD	Human α 3, residues 1 – 205	38	5 (13)	0.13 (0.11 - 0.20)	9 (24)	0.09 (0.03-0.16)	
NOD-DQ8	Human α 3, residues 1 – 205	7	7 (100)	0.17 (0.07 - 0.24)	2 (29)	0.04 (0.03; 0.06)	
ABoDQ8	Human α 3, residues 1 – 205	11	0	–	0	–	
C57BL/6J	Human neuroblastoma cells (SHSY-5Y)	18	18 (100)	3.40 (1.20 – 10.0)	0	–	
C57BL/6J	Rat α 3 β 4-transfected HEK-293 cells	32	29 (91)	0.41 (0.05 – 2.40)	32 (100)	14.0 (3.90 – 22.0)	
C57BL/6J	Non-transfected HEK-293 cells	24	0	–	0	–	

[†] nAChR α 3 subunit sequences are identical in rat and mouse; the major extracellular domain differs from the human sequence at 10 of the 205 residues. Polypeptide (150 μ g) was injected *i.d.*, 1 to 3 times.