Human Monoclonal IgM Anti-Gal(β1-3)GalNAc Autoantibodies
Bind to the Surface of Bovine Spinal Motoneurons

FLORIAN P. THOMAS, M.D., M.A., JEFFREY E. THOMAS, M.D.,
SAUD A. SADIQ, M.D., LEONARD H. VAN DEN BERG,
ROBERT I ROELOFS, M.D., NORMAN LATOV, M.D., PH.D., AND
ARTHUR P. HAYS, M.D.

Abstract. An IgM monoclonal autoantibody (M-protein) with anti-Gal(β1-3)GalNAc activity from a patient with lower motor neuron disease bound to the surface of motoneurons isolated from bovine spinal cord. The Gal(β1-3)GalNAc epitope is shared by the gangliosides GM1 and GD1b and by several glycoproteins in the nervous system, and binding was abolished by preabsorbing the patient's serum with GM1. Antibodies specific for GM1, however, which do not bind to Gal(β1-3)GalNAc, did not bind to the motoneurons. This suggests that Gal(β1-3)GalNAc bearing glycoproteins or glycolipids other than GM1 are expressed on the surface of motoneurons, while GM1 may be absent or shielded, and that antibody binding to the cell surface might contribute to the development of the motor neuron disease.

Key Words: Autoantibodies; Gal(β1-3)GalNAc; GM1 ganglioside; Motoneurons; Motor neuron disease.

INTRODUCTION

Human IgM autoantibodies with anti-GM1 activity are sometimes associated with lower motor neuron disease or predominantly motor neuropathy (1–6). In most cases the anti-GM1 antibodies react with the Gal(β1-3)GalNAc epitope shared by GM1 and GD1b and several glycoproteins in the central and peripheral nervous system (6–8), but in some patients with neuropathy the antibodies are specific for GM1 (unpublished data). Because these antibodies are associated with disorders of the motor unit, we investigated their binding to the surface of isolated bovine motoneurons to determine whether motoneurons might serve as targets for the autoantibodies in vivo.

MATERIALS AND METHODS

Patients and Sera: Serum was obtained from a patient (H.U.) with lower motor neuron disease, whose IgM monoclonal antibodies reacted with the Gal(β1-3)GalNAc epitope of GM1, asialo-GM1 and GD1b (2). Serum was also obtained from a patient (E.K.) with motor neuropathy, who had increased titers of anti-GM1 antibodies that did not crossreact with GD1b, GM2 or Gal(β1-3)GalNAc (unpublished data). As control, serum from a normal individual was used, and in addition the patients' serum was tested for binding following immunoabsorption with GM1 bound to octyl-sepharose CL-4B gel (8). Sera was stored at −70°C.

Isolation of Motoneurons: Motoneurons were isolated by a modification of the method

From the Departments of Pathology (FPT, APH) and Neurology (FPT, JET, SAS, LHvdB, NL), College of Physicians & Surgeons, Columbia University, New York, New York, and the Department of Neurology (RIR) of University of Minnesota Hospital and Clinics, Minneapolis, MN.

Correspondence to: Florian P. Thomas, M.D., M.A., Department of Neurology, College of Physicians & Surgeons of Columbia University, Black Building, Room 323, 630 West 168th Street, New York, NY 10032.

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Fig. 1. Staining of acetylcholine containing bovine motoneurons by choline acetyltransferase immunocytochemistry. × 150.

described by Engelhardt et al (9). The cervical and lumbar anterior horns were removed from 20 fresh bovine spinal cords and placed in an isolation medium containing 32% sucrose, 10 mM Tris and 0.5 mM EDTA, pH 7.4, at a tissue:medium ratio of 1:6 (w:v) and disaggregated by vigorous stirring for two hours. The suspension was filtered three times through a nylon sieve with a pore size of 250 μm, followed by three more filtrations through a sieve with a pore size of 125 μm and brought up to 120 ml with the sucrose solution. It was then fractionated by a discontinuous sucrose density gradient by adding 20 ml of the sample over a gradient of 8 ml 48.5% sucrose overlayed with 6 ml 42% sucrose and 6 ml 33% sucrose (in 10 mM Tris, 0.5 mM EDTA, pH 7.4). Following the first centrifugation in a SW 28 rotor at 20,000 rpm for 30 minutes (min), the pellets were resuspended in 24 ml 20% (w:v) Ficoll 400 and centrifuged again at 20,000 rpm for 30 min. The motoneurons, which formed a layer on the surface of the Ficoll solution, were removed, suspended in phosphate buffered saline (PBS) containing 0.2 M NaCl, 0.05 M NaH₂PO₄, pH 7.4, and centrifuged at 1,500 rpm for ten min. This step was repeated twice. All procedures were done at 4°C.

_Determination of Choline Acetyltransferase (CAT) Activity by Immunocytochemistry:_ 100 μl of the motoneuron suspension in PBS was placed on a glass slide and air dried. The motoneurons were then fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for five min and immunostained with a rat monoclonal anti-CAT antibody (Boehringer-Mannheim, New York) by the peroxidase-antiperoxidase method as described by Burke and Karanas (10).

_Binding of Autoantibody to Motoneurons Following Fixation:_ 100 μl of the motoneuron suspension in PBS was placed on a glass slide, air dried and fixed for four min in 4% paraformaldehyde. They were then washed twice for five min in PBS and exposed to 50% normal goat serum (NGS) in PBS for 20 min. The cells were then immunostained with the patients’ serum at dilutions of 1:100, 1:500 and 1:1,000 in PBS for one hour. After washing twice in PBS for five min, the motoneurons were exposed to affinity purified, fluorescein isothiocyanate (FITC) conjugated goat antibodies (F(ab')₂ fragments) to human IgM at 0.1 μg/μl (Sigma, St. Louis, MO). All procedures were carried out at 4°C. As controls, the primary serum was omitted or it was preabsorbed with GM1 to remove the M-proteins.

_Binding of Human Anti-GM1 Autoantibodies to the Surface of Unfixed Motoneurons:_ Freshly
Fig. 2. Bovine motoneurons, fixed with 4% paraformaldehyde, immunostained with serum at a dilution of 1:100, and with a FITC-conjugated second antibody to human IgM. A. Patient serum; immunofluorescence shows intense patchy or granular IgM deposits on neuronal cell surfaces. Arrows indicate intensely auto-fluorescent lipofuscin within the cytoplasm of motoneurons. The speckled materials represents immunostaining of remnants of motoneurons that were dislodged during the staining procedure. B. Patient serum absorbed with GM1; there is no immunostaining, but only auto-fluorescent lipofuscin. × 300.

isolated, unfixed motoneurons were suspended in 50 μl of PBS, and NGS was added for a final dilution of 1:100. Patient serum was then added for a final dilution of 1:100, and the samples were allowed to incubate for one hour. The cells were then pelleted by centrifugation at 2,000 rpm for ten min, and the pellet was resuspended in PBS containing FITC conjugated...
goat antibody to human IgM at 0.1 μg/μl and incubated for an additional 30 min. The cells were again pelleted by centrifugation for ten min at 1,500 rpm, the pellet was resuspended in 90% glycerin and mounted on glass slides. All steps were done at 4°C. As controls, cells were immunostained with normal serum or with the second antibody alone.

RESULTS

By phase microscopy, the isolated cells were of a shape typical of motoneurons with a long axis of 50 to 100 μm, and they stained positively for choline acetyltransferase, thus confirming their identity (Fig. 1).

The serum IgM from patient H.U. that reacted with the Gal(β1-3)GalNAc epitope of GM1 and GD1b and of several glycoproteins strongly immunostained the cell surface of fixed motoneurons and their processes at dilutions of up to 1:1,000 (Fig. 2A). The staining was granular or patchy. Some staining over the cell body may have been in the cytoplasm as well as on the surface. Arrows indicate intensely autofluorescent material, within the cytoplasm of motoneurons. The speckled material in the field represents immunostaining on remnants of motoneurons that were dislodged during the staining procedure. Binding was abolished following absorption of the patient’s serum with GM1 indicating that the M-protein was responsible for the binding (Fig. 2B). There was also no binding when the serum was omitted. The immunostaining was highly reproducible, and yielded identical results in several separate experiments. By contrast the serum from patient E.K. that reacted with GM1 only, did not immunostain motoneurons even at a dilution of 1:100.

The anti-Gal(β1-3)GalNAc M-protein from patient H.U. also bound to the surface membranes of unfixed motoneurons including their cell processes. The deposits were granular and patchy (Fig. 3A), and they were more distinct than with the fixed cells. No binding was seen with the second antibody alone or with serum from a normal subject, where the only fluorescence observed was the orange autofluorescence of lipofuscin identified in phase contrast of the same motoneurons as high contrast granular material (Fig. 3B, C).

DISCUSSION

These studies indicate that antibodies which react with the Gal(β1-3)GalNAc epitope shared by GM1 and GD1b and by several neural glycoproteins, bind to the surface of spinal motoneurons, whereas antibodies specific for GM1, do not. In previous studies the anti-Gal(β1-3)GalNAc antibodies from patient H.U. immunostained peripheral nerve and both white and gray matter in sections of the spinal cord (11), while the anti-GM1 antibodies from patient E.K. immunostained peripheral nerve and spinal cord white matter only. These observations suggest that GM1 is absent in spinal motoneurons or that it contains epitopes that are inaccessible to autoantibodies. Anti-Gal(β1-3)GalNAc antibodies might therefore cause lower motor neuron disease by binding to glycoproteins or glycolipids other than GM1 on the surface of motoneurons, but they might also cause neuropathy by interacting

Fig. 3. Bovine motoneurons, unfixed, immunostained with serum at a dilution of 1:100 and with a FITC-conjugated second antibody to human IgM. A. Patient serum; immunofluorescence shows intense patchy or granular IgM deposits on cell surfaces. B. Serum from a normal individual; there is no immunostaining, but only intensely autofluorescent lipofuscin within the cytoplasm of two motoneurons. C. Phase contrast demonstrating the lipofuscin in two motoneurons. ×300.
with GM1 or with other crossreactive glycoconjugates in peripheral nerve. Autoantibodies specific for GM1, however, which are associated with motor neuropathy (unpublished data), might exert their effect by binding to GM1 in peripheral nerve only.

The anti-Gal(β1-3)GalNAc antibodies might bind to either glycoproteins or to GD1b on the surface of motoneurons. GD1b is more concentrated in gray matter, whereas white matter contains more GM1 (12), and crossreactive Gal(β1-3)GalNAc bearing glycoproteins are present in both central and peripheral myelin and axons (8). In other investigations of the distribution of gangliosides in the nervous system, tetanus toxin which recognizes GD1b and other gangliosides, binds to the presynaptic region at the neuromuscular junction and to glial and axonal structures at the nodes of Ranvier in the mouse (13, 14). Cholera toxin which reacts specifically with GM1 (15), binds to the pre- and postsynaptic regions in the rat central nervous system (16) and to the nodes of Ranvier in mouse peripheral nerve (17), and both cholera toxin and anti-GM1 antibodies bind to embryonic chick dorsal root ganglia (18).

The Gal(β1-3)GalNAc epitope and GM1 are highly enriched in the central and peripheral nervous system, but there are significant differences in their distribution within the nervous system and between species (11). The effect of anti-GM1 antibodies in vivo might, therefore, in part depend on the fine specificities and crossreactivities of the autoantibodies, and on the species in which they occur. In addition, factors such as local trauma, inflammation or other noxious insults might disrupt the blood–nerve or the blood–brain barrier (19, 20), allowing the antibodies access to otherwise sequestered structures. Injection of rabbit anti-GM1 antibodies into the lumbosacral subarachnoid space of the rat, for example, has been reported to induce demyelination in spinal roots and spinal cord and alterations of astroglia, but did not affect motoneurons (21), possibly because the antibodies were not reactive with Gal(β1-3)GalNAc, or because of species differences in the distribution of the antigen. Further investigation of the fine specificities of anti-GM1 antibodies in patients with neurological disease and characterization of their target epitope and creation of an animal model for the disease, would help elucidate their mechanisms of action.

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REFERENCES

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