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Therapeutics to Promote CNS Repair: A Natural Human Neuron-Binding IgM Regulates Membrane-Raft Dynamics and Improves Motility in a Mouse Model of Multiple Sclerosis

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Abstract

We have discovered a role for natural autoantibodies in central nervous system repair, remyelination and axon protection. These natural human antibodies are of the immunoglobulin M (IgM) isotype, and they bind to the surface of neural cells. The epitope of the antibody includes sialic acid because treatment with sialidase disrupts the binding. A fully human recombinant form of one of these IgMs, rHlgM12, has the same properties as the serum-derived IgM. rHlgM12 enhanced polarized axonal outgrowth from primary neurons when presented as a substrate in vitro and improved motor functions in chronically Theiler's virus-infected SJL mice, a model of MS. rHlgM12 bound to neuronal surfaces and induced cholesterol and ganglioside (GM1) clustering, indicating that rHlgM12 functions through a mechanism of axonal membrane stabilization. Our work demonstrates that a natural human neuron-binding IgM can regulate membrane domain dynamics. This antibody has the potential to improve neurologic disease.

Keywords

CNS repair; IgM; Membrane raft; Multiple sclerosis; Motility

Introduction

Restoring damaged central nervous system (CNS) networks is an integral goal of treating neurologic disease. There is evidence that neuronal and axonal injury ultimately result in permanent neurologic deficits [1]. Development of therapies to protect spared CNS axons and to prevent injured axons from degeneration is critical to limit and prevent permanent disability. Many studies indicate that reparative-signaling cascades are initiated at the cell membrane [2]. Cell membranes are composed of a mixture of cholesterol, sphingolipids, and proteins that are embedded and/or anchored in a bilayer of glycerolphospholipids. Different classes of lipids behave very differently within the membrane. Glycerolphospholipids have a tendency toward mobile fluidity, whereas sphingolipids and cholesterol are more tightly packed. In the physiological membrane, lipid assemblies are segregated into domains of different sizes and functions. The so-called "lipid raft" concept was proposed more than ten

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years ago; these structures are confirmed biophysical entities now commonly referred to as membrane rafts [3, 4]. Membrane rafts are more accurately defined as dynamic, nanoscale, sterol-sphingolipid-enriched, ordered assemblies of protein-lipid organizations. They can be stimulated to coalesce into larger, more stable domains. Membrane rafts are involved in almost every aspect of cell life, including intracellular-molecule/vesicle trafficking, cell division, cell migration/differentiation to synaptic plasticity in the CNS, virus-infection/replication, and T- and B-cell activation and signaling. Therefore, membrane raft signaling molecules are attractive therapeutic targets.

The understanding that membrane rafts may be involved in CNS repair arises from early observations that natural and semi-synthetic glycosphingolipids are neuro-protective. When added to primary cultured neurons, glycosphingolipids protect neurons against excitatory amino acid-induced toxicity [5, 6]. When administered to animals with spinal cord injury, trauma, or brain ischemia, monosialotetrahexosylganglioside (GM1) improved functional recovery [7, 8]. Although gangliosides are essential to the maintenance of membrane raft integrity, [9], gangliosides such as GM1 and its derivatives are antigenic [10]. In autoimmune peripheral neuropathy, especially Guillain-Barre syndrome (GBS), autoantibodies against gangliosides are often present in patient sera [11]. Anti-ganglioside antibodies may be generated following bacterial infections in which the microbial glycans induce immunogenicity by mimicking endogenous gangliosides [10, 12]. Anti-ganglioside antibodies can exacerbate inflammatory destruction of glial and neuronal structures.

Reagents that mimic the action of exogenous gangliosides or that maintain membrane raft stability without the side effects of immunogenicity are attractive candidates as reparative drugs. For example, targeting caveolin-1, a raft molecule, promotes neuronal arborization [13]. The study of ganglioside-deficient mice emphasizes the necessity and protective role of gangliosides in the neuronal membrane; in these mice, neurons degenerate with age [9].

Natural human antibodies are commonly encoded by germline sequences and belong to the normal immunoglobulin repertoire [14, 15]. In contrast to conventional antibodies that are produced to exogenous antigen stimulation, natural antibodies are generated without obvious antigen activation. Many natural antibodies belong to IgM class, have relatively low affinity but high avidity due to their pentameric structure. Avrameas et al. has proposed that natural autoantibodies have natural physiological role and are important for homeostasis [16]. It has been shown that the repertoire of natural antibodies differ between individuals, but within one individual the levels seem to be stable with aging [17]. Here we report an important role for a human natural monoclonal IgM that regulates membrane raft motility, promotes polarized axon outgrowth, and improves functional recovery in a mouse model of multiple sclerosis (MS). We propose that these effects are driven by IgM-mediated membrane raft reorganization.

Materials and Methods

Antibodies

1) Recombinant human IgM 12 (rHIgM12)—Plasmids expressing the heavy and light chain coding sequences for the predominant antibody expressed in the serum of a Waldenstrom's macroglobulinemia patient were co-transfected along with a human J-chain transgene into CHO-S cells (GibcoBRL, cat# 11619). The resulting CHO cells were selected with increasing doses of methotrexate, and a stable clone that produced antibody as measured by ELISA was sub-cloned and expanded. rHIgM12 in culture supernatant was purified by chromatography to 97%, as measured by HPLC analysis.

2) Control human IgM—Non-reactive control human IgMs were selected from a series of serum samples obtained from patients with Waldenstrom's macroglobulinemia, multiple myeloma, or lymphoma. Non-reactivity was established by performing cerebellar slice immunocytochemistry, as reported previously [18].

Cell culture and neurite outgrowth assay

Primary hippocampal neurons were prepared from FVB mice (The Jackson Laboratory, ME). Glass coverslips were coated a thin layer of nitrocellulose membrane and then exposed to solutions of poly-D-lysine (PDL), rHlgM12, or a control human IgM (50 g/ml). Embryonic day 15 hippocampal neurons were dissociated in trypsin-EDTA and plated onto these substrates. Neurons were grown in Neurobasal Medium containing 2% (v/v) B27 supplement and assayed for neurite outgrowth 12–16 hr after seeding. Neurons were fixed with 4% paraformaldehyde and stained with anti- β 3-tubulin antibody. Filamentous actin (F-actin) was labeled with Texas Red phalloidin. Neurite lengths in digital images were measured using Image J software (National Institute of Health, NIH), processed with Excel (Microsoft), and analyzed statistically using Prism (GraphPad) [19].

Immunostaining of primary cultured neurons

E15 hippocampal neurons were seeded on PDL plus laminin (4 μ g/ml)-coated glass cover slips. For immunostaining of intracellular molecules, neurons of 1–3 days in vitro (DIV) were fixed with 4% paraformaldehyde and stained after permeabilization in buffer containing 0.2% Triton X-100. For immunostaining surface molecules, both live and fixed neurons without permeabilization were used. Images were collected using an Olympus upright microscope equipped with a 37-Mb digital SPOT camera and processed with Photoshop (Adobe).

Antibodies and other reagents

Anti- β 3-tubulin (Promega); EDTA, poly-D-lysine, and filipin (Sigma); Anti-Tau1 and anti-Map2 (Millipore); Texas Red phalloidin, cholera toxin B (CTB, Alexa Fluor 594), Neurobasal Medium and B27 (Invitrogen).

Theiler's virus model of demyelination and antibody treatment

SJL/J mice (Jackson Laboratories, Bar Harbor, ME) were housed and bred in Mayo Clinic's animal care facility. Animal care and experimental protocols were approved by Mayo Clinic Institutional Animal Care and Use Committee (IACUC) and Research Committee. Demyelinating disease was induced in 6–8-week-old mice by intracerebral injection of Theiler's murine encephalomyelitis virus (TMEV). A 27-gauge needle delivered 10 μ l of Daniel's strain TMEV containing 2.0×10^5 plaque-forming units. This resulted in >98% incidence of infection with rare fatalities. All animals developed mild encephalitis, which resolved within 2 weeks. Axon injury and loss began 3 months after infection, correlating with neurologic dysfunction. SJL mice (uninfected and 90 days post infection) were treated with a single 200 μ g intraperitoneal or intravenous dose of human antibodies (rHlgM12 or isotype IgM control) dissolved in 0.5ml of PBS or vehicle only.

Spontaneous activity monitoring

Spontaneous locomotor activity was recorded using a Digiscan open-field monitoring system (Accuscan Instruments; Columbus, OH) and Versamax software, v.4.12-1AFE. The apparatus consists of 6 acrylic cages (40×40×30.5cm) supported by a metal frame holding two sets of photo cells. The device measures the number of discrete horizontal and vertical movements by tabulating the number of projected infrared beam interruptions. In all cages, mice were exposed to identical environmental conditions: (a) freely accessible food and

water, (b) a normal 12h light/dark cycle, and (c) 70°F ambient temperature. In each experiment, hyperactive and overweight animals were excluded; the remaining mice were randomized. Groups of 5 SJL mice were placed in each cage, and baseline spontaneous activity was collected over 8 consecutive days. After this period, mice were treated with antibodies or saline and monitored over 8 weeks. Data was collected as the number of beam breaks per 1-hour block. The total horizontal and vertical activities were recorded using Versadat program, v.3.02-1AFE (Accuscan Instruments). The data was presented as ratios of averaged weekly post-treatment nocturnal activity and averaged baseline nocturnal activity (percent change from baseline).

Results

Identification of Natural IgM Antibodies in CNS Repair

Earlier studies in our laboratory indicated that immunization with CNS homogenates or the transfer of anti-serum against CNS homogenates promoted repair of demyelinated axons in the CNS in a mouse model of MS [20]. These studies suggested that it is possible to enhance CNS repair by regulating the immune system. In support of this hypothesis, we identified myelin-binding antibodies from the sera of immunized mice that preserved oligodendrocytes and promoted remyelination [21]. Similar remyelination-promoting antibodies from human sera have subsequently been identified, characterized, and cloned. A recombinant form of one human IgM, rHIgM22, has been generated and is close to phase I clinical trials [22]. Accumulating evidence indicates that myelin not only enables saltatory conduction of action potentials along myelinated axons, but also stabilizes the axolemma. In addition to promoting new myelin, reparative and protective strategies can directly target axons. In a subsequent screening of a sera bank of patients with monoclonal gammopathies, human IgMs (sHIgM) that bound to neuronal surfaces and supported CNS neurite outgrowth were identified [18]. When these human antibodies were screened in vivo, a single peripheral dose of sHIgM12 was found to alter spontaneous activity in mice with long-term demyelination and neurologic dysfunction [22]. The epitope for the antibody involves sialic acid because treatment of neurons with sialidase abolished immunofluorescence staining [18]. A recombinant form of the IgM derived from patient 12, rHIgM12, was constructed. As reported previously, this recombinant human IgM bound to the surface of neurons in a pattern similar to that of the serum-derived IgMs (Fig. 1) [23].

rHIgM12 Regulates Membrane Raft Dynamics

rHIgM12 evenly labeled the neuronal surface when primary neurons were fixed and stained with rHIgM12 without permeabilization (Fig. 1A). In contrast, when live neurons were first treated with bath-applied rHIgM12 for 30 min at 37°C, a reorganization of the membrane raft occurred (Fig. 1B&C). This was shown using markers for membrane cholesterol and GM1, filipin, and cholera toxin B subunit, respectively. rHIgM12 on the neuronal surface aggregated on the membranes (Fig. 1B1&C1). Both cholesterol (Fig. 1B2) and GM1 (Fig. 1C2) were also clustered and co-localized with the aggregated rHIgM12, especially within the growth cone region (Fig. 1B–C, high magnification). These results indicated that rHIgM12 binding to live neurons can regulate membrane-raft dynamics.

rHIgM12 Promotes Axon Outgrowth and Improves Functional Recovery in a Mouse Model of MS

Neurite outgrowth assays were conducted using primary hippocampal neurons. When presented as a substrate, rHIgM12 substantially promoted hippocampal neuron differentiation (Fig. 2). Within 12–16 hours after plating, hippocampal neurons growing on rHIgM12 developed multiple neurites, with one of them much longer compared to the neighboring neurites. In contrast, neurons seeded onto substrates of control human IgMs

were mostly unattached and washed off after fixation (data not shown). Neurons plated on poly-D-lysine (PDL) developed multiple short neurites symmetrically from the cell body (Fig. 2A, B). This indicated that rHIgM12 promoted neuron differentiation. To further study rHIgM12-enhanced axonal outgrowth, neurons on different substrates were fixed and immunolabeled with anti-Tau1 and MAP2 antibodies to identify axons and dendrites, respectively. Tau1 staining was weak in neurons grown on PDL, but was intense and enriched in the distal segment of the longest neurites in neurons plated on rHIgM12 (Fig. 2C, D). MAP2 staining was found primarily in the cell body and the proximal part of all neurites (data not shown). More than 70% of neurons grown on rHIgM12 contained an asymmetric distribution of Tau1 in the distal segments of the longest neurite, whereas less than 20% of neurons grown on PDL presented with similar enrichment of Tau1 (Fig. 2E). These findings suggested that rHIgM12 promoted polarized axonal outgrowth from neurons.

Because rHIgM12 appeared to drive neuronal differentiation, we asked whether the IgM antibody could affect neurologic function, similar to the serum-derived form. TMEV-infected SJL mice were treated with antibodies within 3 months after infection, at a time in disease progression when all animals presented with steadily worsening axonal injury and loss. Virus-infected and uninfected mice were treated with rHIgM12 or non-reactive control human IgM in a single 200 μ g dose dissolved in 0.5 ml of PBS, or with PBS alone. AccuScan activity boxes were used to monitor spontaneous nocturnal activity over the 2 months following treatment (Fig. 2F, G). Improvement in horizontal nocturnal activity was measured in groups of rHIgM12-treated mice, whether the antibody was administered intraperitoneally or intravenously. Activity in two control groups, isotype-control IgM or saline, did not improve (Fig. 2G). As shown previously there was also no change in the performance of rHIgM12-treated, age-matched, uninfected normal mice, indicating that rHIgM12 had no effect on the activity of normal mice (Fig. 2F) [24].

Discussion

The discovery of a human IgM that binds to neural cell membranes and can induce repair indicates that some natural human IgMs can be designed as therapeutic reagents for neurologic disease(s). Natural antibodies bind to self-antigens without obvious antigenic stimuli. They are found in healthy individuals and are usually IgMs. Natural antibodies are polyreactive and can bind to a variety of antigens, including carbohydrates, lipids, nucleic acid, and proteins, or even various combinations of different molecules. They are traditionally regarded as first-line defenders against infectious invasions or as housekeeping molecules to maintain homeostasis in the host. The structural similarity of IgMs and bacterial toxins such as cholera toxin B (CTB) led us to hypothesize that natural IgMs exert their function by cross-linking their membrane targets along with associated signaling molecules.

rHIgM12 binds to neuronal membranes and associates with membrane rafts. rHIgM12 binds evenly to the surface of primary neurons, indicating that the antigen of rHIgM12 is distributed on the outer leaflet of the neuronal membrane. When live neurons remain in the presence of rHIgM12, the IgM reorganizes to aggregate on the cell surface. rHIgM12 is not internalized, even though this phenomenon is often observed with other molecules that bind to the cell membrane, such as CTB or IgMs directed against poly-sialylated neural cell adhesion molecule (PSA-NCAM) [25]. Aggregation of rHIgM12 induces the clustering of cholesterol and ganglioside GM1. These observations demonstrate that rHIgM12 can regulate membrane raft dynamics in live neurons and can coalesce tiny nanoscale membrane rafts into larger membrane domains.

When presented in vitro as a substrate, rHlgM12-induced signaling is amplified and converted into axon induction. We showed that membrane-bound rHlgM12 exists as a complex with tubulin and can regulate microtubule dynamics [25]. This suggests that membrane-cytoskeleton components, which are integral components of membrane rafts, are one of the downstream signaling mediators of rHlgM12 signaling, and that membrane rafts can function as anchors of the cytoskeleton. Clustering of raft molecules enhances the cytoskeleton-membrane interaction that underlies the rHlgM12-induced axon outgrowth.

Whether rHlgM12 alters the membrane organization of neurons and axons in vivo is unknown, although the improvement in neurologic deficits does suggest a change in neuron health. Besides an effect on axons, rHlgM12 may regulate inflammation by facilitating the clearance of inflammatory molecules from apoptotic cells, similar to the proposed mechanism by which natural IgMs facilitate phagocytosis of apoptotic cells [26]. The result of treating with rHlgM12 may be the cumulative effects of multiple actions. In vivo rHlgM12 may interact with its antigens on the axolemma, stabilizing axons and slowing axonal degeneration, or even facilitating regeneration. The IgM molecule may also prevent the progression of inflammation by enhancing clearance of inflammatory molecules released from apoptotic cells or by promoting regulatory T cells. Both mechanisms can facilitate repair of the injured CNS.

It is traditionally regarded that the blood-brain barrier composed by the tight junctions between endothelial cells of brain capillaries segregates the neural tissue from the blood circulation. Accumulating evidences indicate that rather than an immune privileged organ, the CNS is under continuous immune surveillance. We have previously shown that a mouse monoclonal IgM labeled with ³⁵S-methionine crossed the blood brain barrier and entered the CNS [27]. Furthermore, independently from our group, Banks et al. showed that IgM monoclonal antibodies cross the blood brain barrier in a mouse model of Alzheimer's disease [28]. Our laboratory demonstrated previously that these antibodies that cross blood-brain barrier promote remyelination [29, 30] and promote increase in spontaneous activity [24].

Three decades ago, using experimental autoimmune encephalomyelitis (EAE) model Lassmann et al. proposed that certain serum IgM's have a pathogenic role given that when injected in the subarachnoid space induced demyelination [31]. In addition to the evidence for a pathologic role of IgMs in animal models, during the last two decades there were several reports showing a relationship between local oligoclonal IgM synthesis in the cerebrospinal fluid (CSF) and a worse MS course with a long-term disability [32, 33]. This is further supported by the significant beneficial effects of plasma exchange in a subgroup of MS patients with acute demyelinating attacks [34, 35]. In contrast, rHlgM12 is a human, monoclonal neuron-binding antibody present in a serum of a patient in large quantities. Despite large quantities of the antibody, the patient did not show any neurologic or autoimmune disorders. To date rHlgM12 appears to be safe, however formal toxicology experiments in mice and primates need to be performed before the antibody is ready for clinical application.

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Figure 1. rHlgM12 clusters membrane rafts on neuronal membranes

A. DIV1 hippocampal neurons were fixed and stained with rHlgM12. **B–C.** Live DIV3 hippocampal neurons were treated with rHlgM12 at 37°C for 30 min, then fixed without permeabilization and stained with anti-human IgM secondary antibody, filipin (**B**, Cholesterol), or cholera toxin B (**C**, GM1) that labeled cholesterol or ganglioside GM1, respectively. rHlgM12 aggregated on neuronal surfaces (**B1–C1**) and induced the clustering of cholesterol (**B2**) and GM1 (**C2**). **b1–b2** and **c1–c2** show a higher magnification of boxed regions in **B** and **C**. Arrow heads indicate the localization of both aggregated rHlgM12 and cholesterol or GM1. Scale bar, 10 μm.

Figure 2. rHIgM12 promotes polarized axon outgrowth in vitro and improves the horizontal activity of SJL mice in a MS model

Hippocampal neurons were seeded on poly-D-lysine (PDL) (A, C) or rHIgM12 (B, D) substrates that were coated on nitrocellulose membranes attached to glass coverslips. The neurons were fixed and stained 12–16 hours after plating with anti β 3-tubulin (β 3-Tub) antibody to label microtubules (A), or stained with anti-Tau1 antibody to label the axons (C–D). **E.** Compared to the PDL substrate, neurons grown on rHIgM12 showed a higher percentage of axons, with Tau1 asymmetrically enriched in the distal segments. **F–G.** Both normal and 90 days post-TMEV-infection SJL mice were placed in AccuScan activity-monitoring boxes. Baseline measurements were collected over 8 days. After treatment, mice were monitored continuously for 8 weeks. **F.** None of the treatments induced increased horizontal activity in uninfected mice. A gradual decline in activity in all three groups of normal mice may be explained by habituation to the environment. Bars represent the averaged activity over one week divided by the baseline activity. **G.** Regardless of the route of administration, treatment with rHIgM12 improved horizontal spontaneous activity, whereas treatments with control IgM or saline did not show a significant effect. Scale bar, 20 μ m.