N-Acetylglucosamine Inhibits T-helper 1 (Th1)/T-helper 17 (Th17) Cell Responses and Treats Experimental Autoimmune Encephalomyelitis*†‡§¶

Ani Grigorian†§, Lindsey Araujo‡, Nandita N. Naidu¶, Dylan J. Place‡, Biswa Choudhury†, and Michael Demetriou†§‡¶

From the Departments of †Neurology and ‡Microbiology and Molecular Genetics, ¶Institute for Immunology, University of California, Irvine, California 92697 and †Glycotechnology Core Resource, University of California at San Diego, La Jolla, California 92039

Background: Multiple sclerosis (MS) has been linked to genetic and environmental dysregulation of Golgi N-glycosylation. Current treatments and emerging oral therapies for multiple sclerosis (MS) are limited by effectiveness, cost, and/or toxicity. Genetic and environmental factors that alter the branching of Asn (N)-linked glycans result in T cell hyperactivity, promote spontaneous inflammatory demyelination and neurodegeneration in mice, and converge to regulate the risk of MS. The sugar N-acetylglucosamine (GlcNAc) enhances N-glycan branching and inhibits T cell activity and adoptive transfer experimental autoimmune encephalomyelitis (EAE). Here, we report that oral GlcNAc inhibits T-helper 1 (Th1) and T-helper 17 (Th17) responses and attenuates the clinical severity of myelin oligodendrocyte glycoprotein (MOG)-induced EAE when administered after disease onset. Oral GlcNAc increased expression of branched N-glycans in T cells in vivo as shown by high pH anion exchange chromatography, MALDI-TOF mass spectroscopy and FACS analysis with the plant lectin 1-phothoehamagglutinin. Initiating oral GlcNAc treatment on the second day of clinical disease inhibited MOG-induced EAE as well as secretion of interferon-γ, tumor necrosis factor-α, interleukin-17, and interleukin-22. In the more severe 2D2 T cell receptor transgenic EAE model, oral GlcNAc initiated after disease onset also inhibits clinical disease, except for those with rapid lethal progression. These data suggest that oral GlcNAc may provide an inexpensive and nontoxic oral therapeutic agent for MS that directly targets an underlying molecular mechanism causal of disease.

Results: Oral treatment of mice with the sugar N-acetylglucosamine (GlcNAc) enhances N-glycosylation, suppressing inflammatory T cell responses and an MS-like disease when initiated after disease onset.

Conclusion: Disease progression is suppressed by GlcNAc.

Significance: GlcNAc may provide the first MS therapy that directly targets an underlying mechanism causal of disease.

Current treatments and emerging oral therapies for multiple sclerosis (MS) are limited by effectiveness, cost, and/or toxicity. Genetic and environmental factors that alter the branching of Asn (N)-linked glycans result in T cell hyperactivity, promote spontaneous inflammatory demyelination and neurodegeneration in mice, and converge to regulate the risk of MS. The sugar N-acetylglucosamine (GlcNAc) enhances N-glycan branching and inhibits T cell activity and adoptive transfer experimental autoimmune encephalomyelitis (EAE). Here, we report that oral GlcNAc inhibits T-helper 1 (Th1) and T-helper 17 (Th17) responses and attenuates the clinical severity of myelin oligodendrocyte glycoprotein (MOG)-induced EAE when administered after disease onset. Oral GlcNAc increased expression of branched N-glycans in T cells in vivo as shown by high pH anion exchange chromatography, MALDI-TOF mass spectroscopy and FACS analysis with the plant lectin 1-phothoehamagglutinin. Initiating oral GlcNAc treatment on the second day of clinical disease inhibited MOG-induced EAE as well as secretion of interferon-γ, tumor necrosis factor-α, interleukin-17, and interleukin-22. In the more severe 2D2 T cell receptor transgenic EAE model, oral GlcNAc initiated after disease onset also inhibits clinical disease, except for those with rapid lethal progression. These data suggest that oral GlcNAc may provide an inexpensive and nontoxic oral therapeutic agent for MS that directly targets an underlying molecular mechanism causal of disease.

Multiple sclerosis (MS) is characterized by inflammatory demyelination and neurodegeneration producing acute and chronic neurological symptoms. Current treatment strategies for MS are predominated by injectable therapies targeting inflammatory demyelination, including first-line therapies glatiramer acetate (Copaxone; Teva) and β-interferons (Betaferon, Novartis) is the first orally active treatment for MS, but serious adverse effects have been observed (1, 2). Other oral agents are also in development, including cladribine, which has shown efficacy as well as significant toxicity (3). Importantly, these new oral agents broadly and nonspecifically suppress the immune system, a questionable therapeutic approach given our increasing understanding of the pathogenic mechanisms promoting disease. The limitations of current medications highlight the need for safer, more convenient, and less costly treatment strategies. A simple oral therapy that promotes self-tolerance by targeting an underlying disease mechanism, rather than broad and nonspecific immunosuppression, remains a major unmet need for the management of MS.

As a complex trait disease, multiple genetic and environmental factors combine to determine MS susceptibility (4–6). Significant progress in understanding these mechanisms has been made in recent years. Genome-wide association studies have identified a number of genes associated with MS susceptibility, including variants of IL-2 receptor-α (IL2RA, rs2104286) and IL-7 receptor-α (IL7RA, rs6897932) (7–9). MGAT5, a gene encoding an enzyme in the Asn (N)-linked protein glycosylation pathway, was recently identified in a genome-wide association study for variants regulating MS severity (10). We recently demonstrated that the IL2RA and IL7RA MS risk variants alter N-glycan branching by blocking IL-2 and IL-7 signaling-mediated changes in MGAT1, a Golgi gene upstream of MGAT5 (11). Moreover, an MS-associated variant of MGAT1...
interacts with multiple MS modulators to control N-glycan branching and MS risk, including the IL2RA and IL7RA risk variants and vitamin D₃ (11). Vitamin D₃ inversely associates with MS, regulates MGAT1, and inhibits experimental autoimmune encephalomyelitis (EAE) by promoting N-glycan branching (11). Mgat5 deficiency in 129/Sv mice results in enhanced susceptibility to EAE and spontaneous kidney autoimmunity (12). Several mouse strains highly susceptible to EAE (PL/J, SJL, and NOD) display N-glycan branching deficiency in T cells compared with resistant strains (129/Sv, BALB/c, and B10.S) (13). The PL/J strain displays the lowest levels, with a small minority developing spontaneous late onset motor weakness characterized by inflammatory demyelination, neuronophagia, and axonal damage in demyelinated lesions and otherwise normal appearing white matter; phenotypes markedly enhanced by Mgat5⁺/⁻ and Mgat5⁻/⁻ genotypes in a gene dose-dependent manner. N-Glycan branching in neurons directly controls neuronal survival independent of inflammation, as neuron-specific deficiency of MGAT1 in mice results in spontaneous neuronal apoptosis in vivo and neurological deficits (14). Combined, these data suggest that multiple genetic and environmental factors converge to disrupt N-glycan branching, thereby promoting both autoimmune demyelination and neurodegeneration in MS. Therapeutic manipulation of N-glycan branching may provide a simple strategy to correct this underlying molecular defect.

Branching by the Golgi enzymes Mgat1, 2, 4, and 5 allows increased production of N-acetyllactosamine and poly-N-acetyllactosamine, ligands for the galectin family of carbohydrate binding proteins (Fig. 1) (15, 16). Galectins bind N-glycans in proportion to N-acetyllactosamine content, which is enhanced by branching and extension with poly-N-acetyllactosamine. Multivalent binding of galectins to N-glycans attached to surface glycoproteins forms a molecular lattice at the cell surface that regulates the clustering and endocytosis of transmembrane receptors and transporters to control cell growth and differentiation (12, 17–22). In T cells, N-glycan branching inhibits basal and activation signaling through the T-cell receptor (TCR) and CD45, promotes growth arrest by cytotoxic T lymphocyte antigen-4 (CTLA-4), and enhances T-helper 2 (Th2) over T-helper 1 (Th1) differentiation (12, 21, 23–28). Metabolically increasing availability of substrate (i.e., UDP-GlcNAc) to Golgi enzymes by supplementing cells in vitro with the simple sugar N-acetylglucosamine (GlcNAc) enhances N-glycan branching, suppresses T cell growth, promotes CTLA-4 surface expression, and inhibits adoptive transfer EAE (29). GlcNAc also appears to be active when given orally to mice, with FACS analysis using the plant lectin L-PHA indicating enhanced N-glycan branching (29). Indeed, oral GlcNAc suppresses spontaneous autoimmune diabetes in nonobese diabetic mice when initiated prior to disease onset (29). Here, we report that oral GlcNAc has immunomodulatory effects and inhibits EAE when initiated after disease onset, and we confirm increases in N-glycan branching by MALDI-TOF mass spectroscopy and anion exchange chromatography. As GlcNAc is a dietary supplement available “over the counter” in the United States and has been used safely in humans orally (30), our data suggest that oral GlcNAc may provide a simple and inexpensive therapeutic strategy to target an underlying molecular and genetic defect promoting disease.

**EXPERIMENTAL PROCEDURES**

**MALDI-TOF Mass Spectroscopy and High pH Anion Exchange Chromatography (HPAEC)—**MALDI-TOF and HPAEC-FL profiling of N-glycans were performed by the Glycotechnology Core Resource at the Glycobiology Research and Training Center at the University of California, San Diego. CD3⁺ T cells were treated with 20 mM HEPES buffer at pH 8.2 containing 1% SDS at 100 °C for 5 min. The sample was cooled to room temperature, and SDS was blocked with 1.25% Nonidet P-40 at room temperature for 30 min followed by treatment with peptide N-glycosidase F to release the N-glycans. Released N-glycans were further purified by SepPak C18 cartridge and Poly-Graphitized-Charcoal cartridge. Purified N-glycans were characterized by MALDI-TOF mass spectrometry and HPAEC-FL methods. For MALDI mass spectrometry the sample was mixed in a 1:1 ratio with super-DHB matrix and spotted on MALDI plates, and spectra were acquired in the positive mode. Identification of fluorescent labeled N-glycans was also done by HPAEC coupled with an online fluorescent detector (HPAEC-FL). A fluorescence dye 2-amino benzamide (2-AB) was tagged to the reducing end of the N-glycans followed by purification of 2-amino benzamide-labeled glycans by Glyko clean S-cartridge. The labeled N-glycans were dissolved in water and separated using a PA-1 column (4 × 250 mm; Dionex). The eluted peaks were identified by matching with retention times of 2-amino benzamide-labeled N-glycans isolated from RNase B (for high mannose glycans) and fetuin (sialylated glycans). CD3⁺ T cells were harvested from age- and sex-matched littermate wild-type PL/J mice orally treated with GlcNAc (Ultimate Glucosamine, Wellesley Therapeutics) for 7 days by supplementing the drinking water at 0.25 mg/ml. 6 mice were used per group, and the harvested CD3⁺ T cells were pooled prior to analyses. To measure serum GlcNAc concentrations, blood was taken from age- and sex-matched littermate wild-type C57BL/6 control mice or mice orally treated with GlcNAc for 7 days by supplementing the drinking water at 0.25 mg/ml. The samples were spun with a 3K spin filter to remove serum proteins and then analyzed by HPAEC. 4 mice were used for the GlcNAc treatment group and 2 mice for the control group. All procedures and protocols with mice were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

**EAE Induction and Oral GlcNAc Treatment—**EAE was induced by subcutaneous immunization of C57BL/6 wild-type and 2D2 TCR transgenic mice with 100 μg of MOG 35–55 peptide (AnaSpec) emulsified in Complete Freund’s adjuvant (Sigma) containing 4 mg/ml heat-inactivated Mycobacterium tuberculosis (H37RA; Difco) distributed over two spots on the hind flank. All mice received 150 ng of pertussis toxin (List Biological Laboratories) by intraperitoneal injection on days 0 and 2 after immunization. Mice were examined daily for clinical signs of EAE over the next 30–40 days with the observer blinded to treatment conditions. Mice were scored daily in a blinded fashion as follows: 0, no disease; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness.
or paralysis and hindlimb paralysis; 5, moribund or dead. Mice were treated orally with GlcNAc by supplementing the drinking water at 0.25 mg/ml starting on the second day of clinical disease and continued for the duration of the study. Treatment was administered every other day, and oral consumption by all mice was verified by measuring the amount of drinking water left over after each treatment. On average, each mouse drank 4.5–5 ml in volume. The 2D2 TCR transgenic mice have specificity for MOG 35-55 peptide and develop EAE when immunized with the complete immunization protocol of encephalitogenic peptide plus pertussis toxin. This immunization regimen has been reported previously to result in 90% disease incidence and 40% mortality in the 2D2 TCR transgenic mice and 82% disease incidence and 0% mortality in nontransgenic C57BL/6 mice (31). All procedures and protocols with mice were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Statistical analysis and p values for EAE mean clinical scores were determined by the Mann-Whitney test.

Antigen-specific Cell Assays—Splenocytes were isolated from GlcNAc or control-treated mice and cultured in vitro with MOG 35-55 peptide titrated from 0 to 40 μg/ml. Cells were cultured in 24-well plates at a concentration of $3 \times 10^6$ cells ml$^{-1}$ for 72–120 h. Culture medium consisted of RPMI...
1640 medium supplemented with 10% FBS, 2 \(\mu M\) l-glutamine, 100 units ml\(^{-1}\) penicillin/streptomycin, and 2 \(\mu M\) 2-mercaptoethanol.

Flow Cytometry and l-PHA Staining—Cells were washed with FACS buffer (PBS containing 0.1% (w/v) sodium azide and 2% BSA) and stained with anti-CD4 (RM4-5), anti-CD25 (PC61.5), anti-FoxP3 (FJK-16s) from eBioscience and Phaseolus vulgaris leucoagglutinating lectin (l-PHA, 4 \(\mu g/ml\)) from Sigma for 30 min on ice. After incubation, cells were washed twice with FACS buffer and analyzed by FACS.

Cytokine Analysis—Supernatants from splenocyte cultures from immunized, and EAE mice were used for cytokine analysis. Cytokine levels were determined by using FlowCytomix Multiplex kit (eBioscience) according to the manufacturer’s protocol. Results are shown as mean of triplicate or greater values ± S.E.

RESULTS

MALDI-TOF Mass Spectrometry and HPAEC Analysis of N-Glycan Structures—To evaluate the effect of oral GlcNAc on N-glycan branching, GlcNAc was provided to age- and sex-matched littermate mice via their drinking water for 7 days. A concentration of 0.25 mg/ml was used (≈50 mg/kg/day), which has previously been observed to maximally enhance binding of l-PHA (P. vulgaris, leucoagglutinin) to ex vivo T cells. l-PHA is a plant lectin that binds specifically to \(\beta1,6\)-GlcNAc-branched N-glycans produced by Mgat5 and serves as a marker of N-glycan branching (12, 32, 33). HPAEC estimated the serum GlcNAc concentration to be 0.66 ± 0.20 mM with GlcNAc treatment (\(n = 4\); supplemental Fig. 1). N-Glycans were isolated from purified CD3\(^+\) T cells and analyzed by MALDI-TOF mass spectrometry in positive and negative mode, comparing control and GlcNAc-treated cells (Fig. 2, A–D). N-Glycan structures predicted from the mass are shown in the profiles and reveal predominantly high mannose type carbohydrates. These N-glycans are near the beginning of the N-glycan biosynthetic pathway, prior to modification by Mgat1 and downstream branching enzymes. However, a major molecular ion signal at \(m/z\) 1809.86 in positive mode was detected in the GlcNAc-treated sample that was absent in the control sample, a mass consistent with a fucosylated biantennary branched N-glycan. At higher masses in negative mode, GlcNAc therapy also increased the level of an unusual signal at 2423 \(m/z\) (Na\(^+\) adduct), a mass consistent with a monoantennary branched structure containing poly-N-acetyllactosamine and a single sialic acid (Fig. 2, C and D). The presence of sialic acid rules out an alternative biantennary N-glycan terminated by two \(\alpha1,3\)-galactoses, a structure previously described in T cell blasts (34).

The increase in branched N-glycans was further supported by HPAEC analysis of fluorescently tagged N-glycans from the two treatment groups (Fig. 2, E and F). High mannose and sialylated branched N-glycans were assigned based on N-glycan standards from RNase B, IgG, and fetuin and correlated with the structures observed by mass spectrometry. The GlcNAc-treated samples had higher amounts of sialylated branched N-glycans relative to high mannose oligosaccharides. Peak 9 eluted at the same time as monosialylated branched N-glycans from fetuin and IgG, suggesting that this corresponds to the major 2423 \(m/z\) structure in the MALDI-TOF spectrum. These standards also suggest that peak 8 represents the nonsialylated biantennary signal observed at 1810 \(m/z\) (Fig. 2, A and B). Although mass spectroscopy and linkage analysis of individual peaks are required to assign structures to these peaks definitively, the combined data support the conclusion that oral GlcNAc enhanced N-glycan branching and extension with poly-N-acetyllactosamine in T cells in vivo. Indeed, these data are consistent with previously published mass spectrometry data of in vitro GlcNAc supplementation revealing increased branched N-glycans in tumor cells and UDP-HexNAc substrate levels in activated mouse T cells (21, 29).

Oral GlcNAc Begun at Initiation of EAE Inhibits Th1/Th17 Responses—The uptake of GlcNAc into cells is by macropinocytosis, a process that depends on the rate of membrane turnover. Although resting T cells have minimal membrane turnover, rapidly dividing T cell blasts have high membrane turnover; suggesting that GlcNAc may more effectively enhance N-glycan branching in the latter. Indeed, in vitro analysis reveals that GlcNAc increased l-PHA binding in T cell blasts at 4-fold lower concentrations than resting T cells (Fig. 3A).

The above data suggest that for treatment of EAE and MS, myelin-activated T cell blasts (with increased macropinocytosis) should be more responsive to GlcNAc than resting T cells, thereby providing a degree of specificity for disease causal cells. Therefore, we examined the role of in vivo GlcNAc treatment in the regulation of T cells during the initiation phase of EAE in C57BL/6 mice, a mouse model induced by MOG 35–55 with adjuvant to mimic CNS pathology in MS. Oral GlcNAc treatment was initiated at the same time as immunization to determine early effects on T cells. After 14 days, splenocytes harvested from mice treated with GlcNAc had reduced CD25\(^+\) T cells upon restimulation with antigen (Fig. 3, B and C) and promoted development of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) regulatory T cells (Fig. 3D). Oral GlcNAc treatment in vivo also inhibited myelin-antigen induced secretion of Th1 (IFN-\(\gamma\) and TNF-\(\alpha\)) and Th17 (IL-17 and IL-22) cytokines that promote disease (Fig. 3E).

Oral GlcNAc Suppresses Th1/Th17 and Treats EAE When Initiated after Disease Onset—To examine the effect of oral GlcNAc when initiated after development of clinical MOG 35–55–induced EAE, 0.25 mg/ml GlcNAc was provided in the drinking water starting on the second day of clinical disease and continued for the duration of the study. This therapeutic approach significantly reduced clinical symptoms and increased N-glycan branching in T cells as seen by l-PHA staining (Fig. 4, A and B). Consistent with this, a significant reduction in CD25\(^+\) T cells was observed in representative GlcNAc-treated mice taken at the peak of disease compared with control mice (Fig. 4C). Compared with control mice, in vivo treatment with oral GlcNAc after disease onset also suppressed proinflammatory recall responses to the encephalitogenic MOG 35–55 peptide, with significant reductions in secretion of IFN-\(\gamma\), TNF-\(\alpha\), IL-17, and IL-22 (Fig. 4D).

To examine whether oral GlcNAc is effective in more severe forms of disease, the 2D2 TCR transgenic EAE model was utilized (31). Immunization of these mice with MOG 35–55 results
in severe and often fatal EAE. Initiating oral GlcNAc after clinical onset of MOG 35-55-induced EAE in 2D2 TCR transgenic mice or mice treated orally with GlcNAc for 7 days and analyzed by MALDI-TOF mass spectrometry (A–D) or HPAEC (E and F). Oral GlcNAc was administered by supplementing the drinking water at 0.25 mg/ml, with intake confirmed by measuring the amount of drinking water consumed. 6 mice were used per group, and the harvested CD3+ T cells were pooled prior to analyses. The assignment of likely structures in the MALDI-TOF spectrum is based on mass and those in HPAEC on N-glycan standards from RNase B, IgG, and fetuin and correlations with the major structures observed in the MALDI-TOF spectrum. Definitive structure assignment of the latter requires HPAEC-mass spectrometry in-line as well as linkage analysis. Abbreviations for individual sugars are defined in Fig. 1.

**DISCUSSION**

Genetic data in mice and humans provides strong evidence for a role of N-glycan branching in demyelinating disease pathogenesis and disease progression (11–13,17–22). Here, we provide data indicating that oral administration of the simple sugar GlcNAc enhances N-glycan branching while inhibiting CD25+ cells, Th1 and Th17 cytokines, and disease progression in EAE when initiated after disease onset. This result is consistent with earlier *in vitro* GlcNAc supplementation data demonstrating suppression of TCR signaling, T cell proliferation, CTLA-4 endocytosis, Th1 differentiation, and adoptive transfer EAE (21, 29). A pilot study of oral GlcNAc in pediatric treatment-resistant inflammatory bowel disease further reveals
the potential of GlcNAc as a therapeutic agent in humans (35). Remarkably, this study reported that 8 of 12 children with severe inflammatory bowel disease went into clinical remission with evidence of histological improvement. Indeed, 3 of the responders relapsed within 1 month following disruption of GlcNAc therapy, but improved again once GlcNAc therapy was reinitiated. Together, these data provide compelling evidence for GlcNAc as a potential simple and cost-effective oral therapy for MS and the need for a large clinical trial to test this hypothesis. If proven effective, it would provide the first therapy that directly targets an underlying genetic mechanism promoting disease.

The molecular mechanism underlying the therapeutic effect of GlcNAc has previously been investigated (20, 21, 29). GlcNAc-mediated inhibition of TCR signaling, T cell proliferation, Th1 differentiation, and CTLA-4 endocytosis are reversed by Golgi inhibitors that block N-glycan branching (i.e. swainsone, deoxymannojirimycin) (29), confirming that GlcNAc alters T cell function by enhancing N-glycan branching.

Like GlcNAc, glucosamine (GlcN) may also supplement the hexosamine pathway to increase UDP-GlcNAc and N-glycan branching while inhibiting autoimmunity (29, 36, 37). GlcN enters the cell through glucose transporters, allowing a 200–400-fold reduction in effective concentration relative to GlcNAc. However, GlcN 6-phosphate also may flux into the glycolytic pathway for ATP production following deamination by glucosamine-6-phosphate deaminase (Fig. 1) (38, 39). In contrast, GlcNAc does not enter glycolysis, the pentose phosphate pathway or the TCA cycle, and is exclusively salvaged into the hexosamine pathway for production of UDP-GlcNAc (40).
Autophagy and endoplasmic reticulum-associated degradation may further recycle GlcNAc within cells. In this regard, the effectiveness of GlcN in increasing N-glycan branching is limited compared with GlcNAc. Indeed, our previous in vitro studies revealed that increasing concentrations of GlcN initially increased but then reduced N-glycan branching (29). The latter likely arises from GlcN 6-phosphate acting as a potent inhibitor of glutamine fructose-6-phosphate amidotransferase, the rate-limiting enzyme shunting fructose 6-phosphate into the hexosamine pathway (41). In contrast to GlcN, increasing GlcNAc concentrations in vitro is observed to only enhance N-glycan branching.

The estimated in vivo serum concentrations of GlcNAc achieved in our experiments (0.66 ± 0.20 mm) appear to be significantly less than that required in vitro to enhance N-glycan branching. Consistent with our in vitro data, Shoji et al. have reported ~25% tissue incorporation/~75% elimination of oral N-[14C]acetylglucosamine (42). The in vivo concentrations of GlcNAc during EAE are likely to be less than those required in vitro for several reasons. First, uptake of GlcNAc via macropinocytosis is enhanced in T cell blasts, which allows myelin antigen-activated T cells to be more sensitive than other cells during EAE. Second, time of exposure to GlcNAc enhances uptake by macropinocytosis. In vitro exposure of GlcNAc is limited to 3–5 days due to increasing T cell death in culture, an issue avoided in vivo. Thus, longer term GlcNAc exposure is only possible in vivo, further lowering the concentration required to enhance N-glycan branching. Consistent with this, N-glycan branching in resting T cells does not increase until after ~7 days of therapy. Finally, the mg/kg doses of GlcNAc that achieve maximal increases in N-glycan branching in vivo also suppress autoimmunity in mice and inflammatory bowel disease in humans (29, 35). This provides a strong correlation between oral GlcNAc supplementation, increases in N-glycan branching in vivo and suppression of EAE and autoimmunity; results supported by genetic data demonstrating that N-glycan

![FIGURE 4. Oral GlcNAc treatment attenuates the clinical course of EAE. A, EAE was induced in C57BL/6 mice by immunization with MOG 35-55 peptide emulsified in Complete Freund’s adjuvant and pertussis toxin. Mice were treated orally with GlcNAc by supplementing the drinking water at 0.25 mg/ml starting on the second day after disease onset and continued for the duration of the study (n = 9 per control group, n = 7 per GlcNAc group). Day 1 indicates the first day of disease onset. Mice were examined daily for clinical signs of EAE over the next 30 days with the observer blinded to treatment conditions and scored daily as follows: 0, no disease; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness or paralysis and hindlimb paralysis; 5, moribund or dead. Mean clinical scores per group daily were compared by the Mann-Whitney U test. B, GlcNAc treatment increased N-glycan branching in T cells as seen by L-PHA staining in representative mice taken at the peak of disease. The results are representative of at least three mice compared from each group. C, a significant reduction in CD25+ T cells was observed in representative GlcNAc-treated mice taken at the peak of disease compared with control mice. The results are representative of at least three mice compared from each group. D, in vivo treatment with GlcNAc inhibited production of proinflammatory cytokines IFN-γ, TNF-α, IL-17, and IL-22 upon restimulation with MOG 35-55 peptide in vitro. p values in D were determined by t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Error bars represent the means ± S.E. of duplicate or greater values unless otherwise stated.](https://www.jbc.org/content/286/46/40139)
Regulation of Th1/Th17 Responses and EAE by GlcNAc

GlcNAc is readily available over the counter in North America and has been used orally as a dietary supplement for many years without reported adverse effects. Large dose intravenous GlcNAc administration demonstrated no toxicity issues and no alterations in blood glucose concentrations or insulin resistance (30). Chronic systematic toxicological studies up to 104 weeks in animals have further validated GlcNAc as a safe compound with no associated toxicity, including lack of increased cancer risk (43, 44). As polymerized GlcNAc forms chitin present in insects and crustaceans, GlcNAc is highly abundant. GlcNAc can be used alone or in addition to other immunoregulatory drugs to increase efficacy and decrease dosage and toxicity. As GlcNAc may provide the first therapeutic approach that directly targets an underlying disease causal mechanism, human clinical trials are warranted.

Acknowledgments—N-Acetylg glucosamine was provided by Wellesley Therapeutics at no cost. N-Glycan composition analysis was performed by the Glycotechnology Core Resource at the University of California, San Diego. We thank Amy Paulino and David Chen for technical assistance.

REFERENCES

1. Cohen, J. A., Barkhof, F., Comi, G., Hartung, H. P., Khatiri, B. O., Montalban, X., Pelletier, J., Capra, R., Gallo, P., Izquierdo, G., Tiel-Wilck, K., de Vera, A., Jin, J., Stites, T., Wu, S., Aradhye, S., and Kappos, L. (2010) N. Engl. J. Med. 362, 402–415

2. Kappos, L., Radue, E. W., O’Connor, P., Polman, C., Hohlfeld, R., Calabresi, P., Selmayr, K., Agoropoulou, C., Leyk, M., Zhang-Auberson, L., and Burtin, P. (2010) N. Engl. J. Med. 362, 387–401

3. Giovannoni, G., Comi, G., Cook, S., Rammohan, K., Rieckmann, P., Soelberg Sorensen, P., Verversch, P., Chang, P., Hamlett, A., Musch, B., and Greenberg, S. J. (2010) N. Engl. J. Med. 362, 416–426

4. Baranzini, S. E., Mudge, J., van Velkinburgh, J. C., Khankhanian, P., Khrebtukova, L., Miller, N. A., Zhang, L., Farmer, A. D., Bell, C. J., Kim, R. W., May, G. D., Woodward, J. E., Cailler, S. I., McElroy, P. J., Gomez, R., Pando, M. J., Clendenen, L. E., Ganusova, E. E., Schilkley, F. D., Ramaraj, T., Khan, O. A., Huntley, I. J., Luo, S., Kwoy, P. Y., Wu, T. D., Schroth, G. P., Oksenberg, J. R., Hauser, S. L., and Kingsmore, S. F. (2010) Nature 464, 1351–1356

5. Ebers, G. C., Bulman, D. E., Sadovnick, A. D., Pady, D. W., Warren, S., Hader, W., Murray, T. J., Seland, T. P., Duquette, P., Grey, T., et al. (1986) N. Engl. J. Med. 315, 1638–1642

6. Ebers, G. C., Sadovnick, A. D., and Risch, N. J. (1995) Nature 377, 150–151

7. Gregory, S. G., Schmidt, S., Seth, P., Oksenberg, J. R., Hart, J., Prokop, A., Cailler, S. J., Ban, M., Goris, A., Barcellos, L. F., Lincoln, R., Maulea, J., Sawcer, S. J., Compston, D. A., Dubois, B., Hauser, S. L., Garcia-Blanco, M. A., Pericak-Vance, M. A., and Haines, J. L. (2007) Nat. Genet. 39, 1083–1091

8. Lundmark, F., Duvefelt, K., Iacobaeus, E., Kockum, I., Wallström, E., Khadem, M., Oturai, A., Ryder, L. P., Saarela, J., Harbo, H. F., Celius, E. G., Salter, H., Olsson, T., and Hellert, J. (2007) Nat. Genet. 39, 1108–1113

9. Hafer, D. A., Compston, A., Sawcer, S., Lander, E. S., Daly, M. J., De Jager, P. L., van Bakker, P. I., Gabriel, S. B., Mirel, D. B., Ivinson, A. J., Pericak-Vance, M. A., Gregory, S. G., Rioux, J. D., McCarthy, I. L., Haines, I. L., Barcellos, L. F., Cree, B., Oksenberg, J. R., and Hauser, S. L. (2007) N. Engl. J. Med. 357, 851–862

10. Brynedal, B., Wojcik, J., Esposito, F., Debaelle, V., Yaouanq, J., Martinelli-Boneschi, F., Edan, G., Comi, G., Hillert, J., and Abderrahim, H. (2010) J. Neuroimmunol. 220, 120–124

11. Mkhikian, H., Grigorian, A., Li, C. F., Chen, H. L., Newton, B., Zhou, R. W., Beeton, C., Torossian, S., Tatarian, G. G., Lee, S. U., Lau, K., Walker, E., Siminovich, K. A., Chandy, K. G., Yu, Z., Dennis, J. W., and Demetriou, M.
Regulation of Th1/Th17 Responses and EAE by GlcNAc

(2011) Nat. Commun. 2, 234
12. Demetriou, M., Granovsky, M., Quaggin, S., and Dennis, J. W. (2001) Nature 409, 733–739
13. Lee, S. U., Grigorian, A., Pawling, J., Chen, I. J., Gao, G., Mozaffar, T., McDermit, C., and Demetriou, M. (2007) J. Biol. Chem. 282, 33725–33734
14. Ye, Z., and Marth, J. D. (2004) Glycobiology 14, 547–558
15. Schachter, H. (1991) Glycobiology 1, 453–461
16. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
17. Partridge, E. A., Le Roy, C., Di Guglielmo, G. M., Pawling, J., Cheung, P., Granovsky, M., Nabi, I. R., Wrana, J. L., and Dennis, J. W. (2004) Science 306, 120–124
18. Brewer, C. F., Miceli, M. C., and Baum, L. G. (2002) Curr. Opin. Struct. Biol. 12, 616–623
19. Ahmad, N., Gabius, H. J., André, S., Kaltner, H., Sabesan, S., Roy, R., Liu, B., Macaluso, F., and Brewer, C. F. (2004) J. Biol. Chem. 279, 10841–10847
20. Grigorian, A., Torossian, S., and Demetriou, M. (2009) Immunol. Rev. 230, 232–246
21. Lau, K. S., Partridge, E. A., Grigorian, A., Silvescu, C. I., Reinhold, V. N., Demetriou, M., and Dennis, J. W. (2007) Cell 129, 123–134
22. Morgan, R., Gao, G., Pawling, J., Dennis, J. W., Demetriou, M., and Li, B. (2004) J. Immunol. 173, 7200–7208
23. Toccano, M. A., Bianco, G. A., Ilerregui, J. M., Croci, D. O., Correale, J., Hernandez, J. D., Zviriner, N. W., Poirier, F., Riley, E. M., Baum, L. G., and Rabinovich, G. A. (2007) Nat. Immunol. 8, 825–834
24. Motran, C. C., Molinder, K. M., Liu, S. D., Poirier, F., and Miceli, M. C. (2008) Eur. J. Immunol. 38, 3015–3027
25. Zhu, C., Anderson, A. C., Schubart, A., Xiong, H., Inomoto, J., Khoury, S. I., Zheng, X. X., Strom, T. B., and Kuchroo, V. K. (2005) Nat. Immunol. 6, 1245–1252
26. Chen, I. J., Chen, H. L., and Demetriou, M. (2007) J. Biol. Chem. 282, 35361–35372
27. Chen, H. L., Li, C. F., Grigorian, A., Tian, W., and Demetriou, M. (2009) J. Biol. Chem. 284, 32454–32461
28. Grigorian, A., Lee, S. U., Tian, W., Chen, I. J., Gao, G., Mendelsohn, R., Dennis, J. W., and Demetriou, M. (2007) J. Biol. Chem. 282, 20027–20035
29. Levin, R. M., Krieger, N. N., and Winzler, R. J. (1961) J. Lab. Clin. Med. 58, 927–932
30. Bettelli, E., Pagany, M., Weiner, H. L., Linington, C., Sobel, R. A., and Kuchroo, V. K. (2003) J. Exp. Med. 197, 1073–1081
31. Cummings, R. D., and Kornfeld, S. (1982) J. Biol. Chem. 257, 11230–11234
32. Grigorian, A., and Demetriou, M. (2010) Methods Enzymol. 480, 245–266
33. Comelli, E. M., Sutton-Smith, M., Yan, Q., Amado, M., Panic, M., Gilimartin, T., Wihesent, T., Lanigan, C. M., Head, S. R., Goldberg, D., Morris, H. R., Dell, A., and Paulson, J. C. (2006) J. Immunol. 177, 2431–2440
34. Salatore, S., Heuschkel, R., Tomlin, S., Davies, S. E., Edwards, S., Walker-Smith, J. A., French, L., and Murch, S. H. (2000) Aliment. Pharmacol. Ther. 14, 1567–1579
35. Ma, L., Rudert, W. A., Harnaha, J., Wright, M., Machen, J., Lamory, K., Qian, S., Lu, L., Robbins, P. D., Trucco, M., and Giannoukakis, N. (2002) J. Biol. Chem. 277, 39343–39349
36. Zhang, G. X., Yu, S., Gran, B., and Rostami, A. (2005) J. Immunol. 175, 7202–7208
37. Wolosker, H., Kline, D., Bian, Y., Blackshaw, S., Cameron, A. M., Fralich, T. L., Schnaar, R. L., and Snyder, S. H. (1998) FASEB J. 12, 91–99
38. Zhang, J., Zhang, W., Zou, D., Chen, G., Wan, T., Li, N., and Cao, X. (2003) J. Cell. Biol. 88, 932–940
39. Wellen, K. E., Lu, C., Mancuso, A., Lemons, J. M., Ryczko, M., Dennis, J. W., Rabinowitz, J. D., Coller, H. A., and Thompson, C. B. (2010) Genes Dev. 24, 2784–2799
40. Broschat, K. O., Gorka, C., Page, J. D., Martin-Berger, C. L., Davies, M. S., Huang, H. C., Guv, E. A., Salsgiver, W. J., and Kasten, T. P. (2002) J. Biol. Chem. 277, 14764–14770
41. Shoji, A., Iga, T., Inagaki, S., Kobayashi, K., Matahira, Y., Sakai, K. (1999) Chitin Chitosan Res. 5, 34–42
42. Lee, K. Y., Shibutani, M., Takagi, H., Arimura, T., Takigami, S., Uneyama, C., Kato, N., and Hirose, M. (2004) Food Chem. Toxicol. 42, 687–695
43. Takahashi, M., Inoue, K., Yoshida, M., Morikawa, T., Shibutani, M., and Nishikawa, A. (2009) Food Chem. Toxicol. 47, 462–471

NOVEMBER 18, 2011•VOLUME 286•NUMBER 46
JOURNAL OF BIOLOGICAL CHEMISTRY 40141