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Glucosamine Abrogates the Acute Phase of Experimental Autoimmune Encephalomyelitis by Induction of Th2 Response

Guang-Xian Zhang,[1] Shuo Yu, Bruno Gran, and Abdolmohamad Rostami

Glucosamine, a natural glucose derivative and an essential component of glycoproteins and proteoglycans, has been safely used to relieve osteoarthritis in humans. Recent studies have shown that glucosamine also possesses immunosuppressive properties and is effective in prolonging graft survival in mice. Whether this reagent is effective in human multiple sclerosis (MS), an inflammatory demyelination in the CNS, is not known. We thus investigated the therapeutic effect of glucosamine on experimental autoimmune encephalomyelitis (EAE), an animal model of MS. We demonstrated that oral, i.p., or i.v. administration of glucosamine significantly suppressed acute EAE, with reduced CNS inflammation and demyelination. A significant, albeit not strong, blockade of Th1 response and an up-regulation of Th2 cytokines (IL-5 and IL-10) are observed in the splenocytes of glucosamine-treated mice. Glucosamine also regulates IL-5 and IL-10 in vitro. As glucosamine is able to effectively suppress acute EAE, has low or absent toxicity, and has been safely used in humans orally, our study suggests a potential use for this drug alone or in combination with other disease-modifying immunotherapies to enhance their efficacy and reduce their doses in MS and possibly other autoimmune disorders. Furthermore, because glucosamine functions not simply as an immunosuppressant, but as a mild immunomodulator, administration of glucosamine provides a novel immunoregulatory approach for autoimmune disorders. ***The Journal of Immunology,*** **2005, 175: 7202–7208.**

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Experimental autoimmune encephalomyelitis (EAE)[2] is a $CD4^+$ T cell-mediated demyelinating autoimmune disease of the CNS (1), which has many similarities to multiple sclerosis (MS) in humans and serves as a model system of this disease. EAE in rodents is characterized by histological lesions in the CNS, typically consisting of demyelination and infiltrates of autoreactive T cells and other mononuclear cells (1, 2). Autoreactive T cells directed against myelin Ags produce high levels of Th1/proinflammatory cytokines IFN-γ, TNF-α, and IL-17 (3–5), while resistance to, or recovery from, the disease is mediated through Th2/regulatory T (Tr) cells producing cytokines IL-4, IL-5, IL-10, among others (4, 6, 7).

Glucosamine is a naturally occurring sugar that is synthesized by virtually all cells with various physiological properties. Recently, glucosamine has been clinically used to treat human osteoarthritis (8, 9), based on its reported beneficial effect in the reconstruction of joint cartilage (8, 9). In addition, the potential immunoregulatory capacity of glucosamine has also been suggested. Indeed, glucosamine has been shown to suppress proinflammatory cytokine action in human chondrocytes (10), to inhibit NF-κB activation and IL-1β bioactivity in rat chondrocytes (11), and to suppress unprimed T cell response by interfering with functions of APCs and by a direct inhibitory effect on CD3-induced T cell proliferation (12). Furthermore, the addition of glucosamine to immune cells in vitro prevented both their activation and their ability to initiate the MLR. More importantly, a single daily i.v. injection of glucosamine was able to prolong cardiac allograft survival in mice (13).

In the present study, we have investigated the therapeutic potential of glucosamine in EAE. We demonstrated that oral, i.p., or i.v. administration of glucosamine significantly suppressed EAE in C57BL/6 mice at the acute phase, but not the chronic phase, with reduced inflammatory infiltration in the CNS. Possible mechanisms underlying the immunosuppression of acute EAE are also discussed.

Materials and Methods

Mice and Ag

Female C57BL/6 mice, 8–10 wk of age, were purchased from The Jackson Laboratory. All mice were housed in the Thomas Jefferson University animal care facilities. Peptide 35–55 of myelin-oligodendrocyte glycoprotein (MOG35–55) (MEVGWYRSPFSRVVHLYRNGK) was synthesized at the Protein Chemistry Laboratory of the University of Pennsylvania by HPLC purification, with a purity of >98%.

Induction of EAE and glucosamine treatment

Mice were each injected s.c. with 200 μg of MOG35–55 in CFA containing 4 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco) over two sites at the back. All mice received 75 ng of pertussis toxin (List Biological Laboratory) by i.v. injection on days 0 and +2 postimmunization (p.i.). Glucosamine (Sigma-Aldrich) dissolved in PBS was fed or injected i.p. or i.v. daily at various doses starting at different time points p.i. EAE was scored as follows (14): 1, limp tail or waddling gait with tail tonicity; 2, waddling gait with limp tail (ataxia); 2.5, ataxia with partial limb paralysis; 3, full paralysis of one limb; 3.5, full paralysis of one limb with partial paralysis of second limb; 4, full paralysis of two limbs; 4.5, moribund; and 5, death. Mice were examined daily in a blind fashion for signs of EAE. All work was performed in accordance with the Thomas Jefferson University guidelines for animal use and care.

Histopathology

On days 23 and 33 p.i., mice were extensively perfused, a piece (2 cm) of spinal cord at the thoracic section was harvested from each mouse for histology, and the remaining part was used for flow cytometry (see below). Sections (5 μm) of spinal cords were stained with H&E or Luxol fast blue (myelin stain). Slides were assessed in a blind fashion for inflammation and

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[2] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; DC, dendritic cell; LDH, lactate dehydrogenase; MNC, mononuclear cell; MOG35–55, peptide 35–55 of myelin-oligodendrocyte glycoprotein; MS, multiple sclerosis; p.i., postimmunization; Tr, regulatory T.

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demyelination, as follows (15). For inflammation: 0, none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into the adjacent tissue. For demyelination: 0, none; 1, rare foci; 2, a few areas of demyelination; and 3, large (confluent) areas of demyelination.

Isolation of CNS cells and flow cytometry

Mononuclear cells (MNCs) from the CNS of MOG35–55-immunized mice were isolated by Percoll gradient centrifugation, as previously described (16), at the peak of clinical disease (day 23 p.i.). Briefly, mice were sacrificed and transcardially perfused with ice-cold GKN solution (2 g of D-(+)glucose, 0.4 g of KCl, 8 g of NaCl, 3.56 g of $Na_2HPO_4 12H_2O$, and 0.78 g of $NaH_2PO_4 2H_2 0$ in 1 L (pH 7.4)) (16) with 2 U/ml heparin (Sigma-Aldrich). Spinal cords were removed into GKN/0.02% BSA (w/v), mechanically dissociated through a 100-μm cell strainer, and enzymatically digested by incubation with 250 μg/ml collagenase/dispase and 250 μg/ml DNase I (Roche) at 37°C for 20–30 min. The digested CNS preparation was washed with GKN/BSA, and the pellet was fractionated on a 70/37/30% Percoll gradient. MNCs were recovered from the 37/70 interface, washed, and resuspended in RPMI 1640 with 10% FCS. Pooled cells (2×10^5/tube) were washed in FACS buffer. After blocking with CD16/32, cells were incubated with Abs to murine CD11b, CD11c, and CD45 (all from BD Pharmingen). Production of TNF-α and IL-10 was analyzed by intracytoplasmic staining of CNS-derived cells with Abs to murine cytokines. MOG35–55 at 25 μg/ml was added to activate CNS-derived cells for 4 h in vitro in the presence of the protein transport inhibitor brefeldin A. Data were acquired on a FACSCalibur (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Lymphocyte proliferative response

Splenocytes were isolated and suspended in complete RPMI 1640 culture medium at a density of 2×10^6/ml. Triplicate aliquots (200 μl) of MNC suspensions were applied to 96-well round-bottom microtiter plates (Nunc). Aliquots (10 μl) of MOG35–55 were added to appropriate wells at a final concentration of 10 μg/ml, Con A at 5 μg/ml, and without Ag/mitogen. After 60 h of incubation, the cells were pulsed for 12 h with 1 μCi of [^{3}H]methylthymidine (sp. act., 42 Ci/mmol). Cells were harvested on fiberglass filters, and thymidine incorporation was measured at a scintillation counter. The results were expressed as the cpm from culture in the presence of Ag, and without Ag.

Cytokine profiles

Splenocytes were cultured at a cell density of 2×10^6/ml in medium without Ag or containing MOG35–55 (10 μg/ml). Supernatants were collected after 48 h and kept at −70°C. Quantitative ELISA for cytokines of Th1 (IFN-γ, IL-17) and Th2/Tr (IL-4, IL-5, IL-10) were performed using paired mAbs, according to the manufacturer's recommendations (BD Pharmingen).

In vitro effect of glucosamine on production of cytokine and NO

To directly address the effect of glucosamine on the function of peripheral mononuclear cells of EAE mice, splenocytes were isolated from EAE mice on day 12 p.i., at the onset of EAE. These cells were cultured at a concentration of 2×10^6/ml in the presence of autoantigen MOG35–55 (10 μg/ml), mitogen Con A (5 g/ml), and without Ag/mitogen. To evaluate the effect of glucosamine on the production of cytokines from the stimulated macrophages, freshly prepared glucosamine (pH 7.4) was added at concentrations of 0, 1, 10, 100, and 1000 μM at the same time of Ag/mitogen stimulation. Supernatants were collected 48 h after culture and assayed using ELISA kits for IFN-γ, IL-17, IL-5, and IL-10 (BD Pharmingen). NO production was estimated from the concentration of nitrite in the culture supernatant using the Greiss reaction (17). The lactate dehydrogenase (LDH) release assay was used to determine cell viability (18).

Statistics

Mann-Whitney *U* test was used for comparison of average clinical scores, Kolmogorov-Smirnov statistics were used for the comparison of flow cytometry data, and ANOVA for other parameters among different groups. All tests were two sided.

Results

Glucosamine alleviated the severity of EAE at the acute, but not chronic phase

To evaluate the effect of glucosamine on MOG-induced EAE in C57BL/6 mice, we administered glucosamine orally, i.p., or i.v. daily in these mice at different time points after induction of EAE. As shown in Fig. 1*A*, all PBS-fed control mice developed chronic-progressive EAE. When glucosamine was fed starting from day 0 p.i., EAE was significantly suppressed at all doses used (10, 50, and 100 mg/day/mouse), with an optimal dose of 10 mg/day/mouse ($p < 0.01$). This dose is also effective i.p. and is optimal in other models (13, 19), including allograft survival (13, 19). The acute disease was also suppressed when glucosamine was administered at 10 mg/mouse/day i.p. starting from the beginning of EAE induction (day 0 p.i.) or from EAE onset (day 13 p.i.) (both $p < 0.01$; Fig. 1*B*). However, EAE severity in both glucosamine-treated groups reached a similar level as in PBS-injected mice at the chronic phase (e.g., day 33 p.i.) even though the treatment continued (Fig. 1*B*). To investigate whether a higher dose would have a more effective suppression, the dose was increased up to 100 mg/day/mouse i.p. and the suppressive effect of this drug was not enhanced (data not shown). The administration of glucosamine starting from day 18 p.i., when EAE was fully developed, had no suppressive effect (Fig. 1*B*). Similar results were also observed when glucosamine was administered i.v. (Fig. 1, *C* and *D*).

Glucosamine suppressed pathological signs of acute EAE

Histological examination of CNS tissues revealed a dramatic difference between glucosamine- and PBS-treated mice at the acute phase of EAE, consistent with the clinical finding. Shown in Fig. 2 are inflammation and demyelination scores from mice receiving PBS or glucosamine at 10 mg/day i.p. starting at different time points p.i. and sacrificed on day 23 p.i. In PBS-treated mice, multiple inflammatory foci were observed in the white matter of the spinal cord, and the infiltration score was 2.7 ± 0.5, with a similar level of demyelination. By contrast, significantly fewer inflammatory cells and little demyelination were detected in glucosamine-treated mice starting from days 0 p.i. and 13 p.i. (both $p < 0.01$). No significant difference was found between PBS- and glucosamine-treated mice starting from day 18 p.i. (Fig. 2). Also, no difference was found for all these groups when mice were sacrificed at the chronic phase of EAE (day 33 p.i.; data not shown).

Cytokine production of APCs isolated from the CNS

To determine the effect of glucosamine on the cytokine productivity of APCs isolated from the CNS, we sacrificed mice at the peak of disease (day 23 p.i.) and performed intracellular staining to define the capacity of CD11b$^+$ cells (resident microglia/infiltrating macrophages) to produce TNF-α, a proinflammatory cytokine involved in the pathogenesis of EAE (3, 4). The average numbers of cells per mouse were as follows: PBS-i.p. control EAE mice, 9×10^5 cells; glucosamine-i.p. from day 0 p.i., 3.1×10^5; glucosamine-i.p. from day + 13 p.i., 4.4×10^5; glucosamine-i.p. from day + 18 p.i., 8.2×10^5. Shown in Fig. 3 are viable cells (gated based on forward and side scatter parameters). Compared with PBS-treated EAE mice, significantly lower numbers of TNF-α-producing cells were found in mice treated with glucosamine at the beginning of immunization (day 0 p.i.; $p < 0.01$) and at EAE onset (day 13 p.i.; $p < 0.01$) than in PBS-treated mice, but not after the disease had developed (day 18 p.i.). Although it has been found that dendritic cell (DC)-like cells (CD11c$^+$) may play a different role from CD11c$^-$ microglia in CNS inflammatory demyelination (20–22), we did not find a difference between these groups for the number of CD11c$^+$ cells that produced TNF-α (Fig. 3). There was no difference in the number/percentage of CD11b$^+$ cells and TNF-α-producing cells between these groups when mice were sacrificed at the chronic phase of EAE (data not shown).

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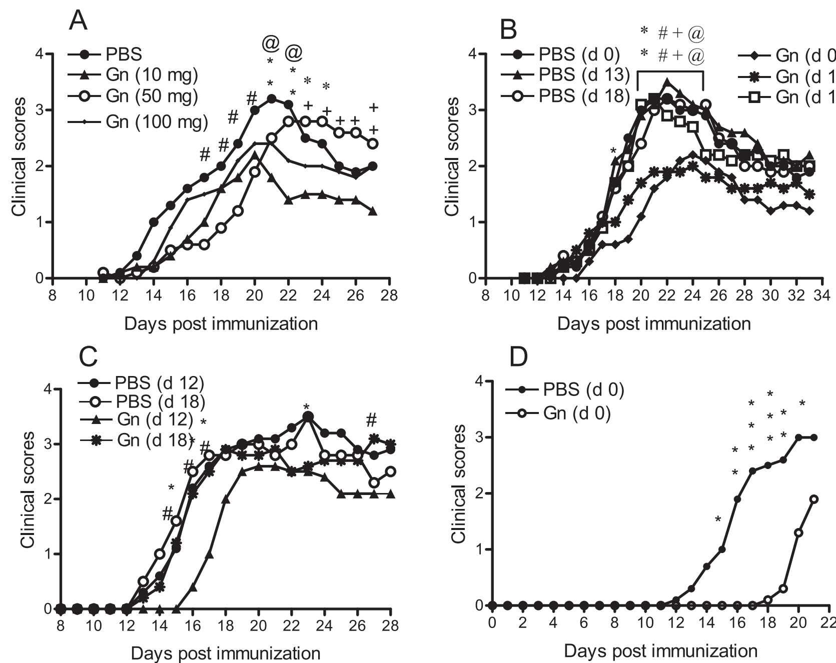

FIGURE 1. Oral, i.p. and i.v. administration of glucosamine suppressed EAE at the acute, but not chronic phase. C57BL/6 mice were immunized with MOG35–55 + CFA. Pertussis toxin was injected on days 0 and 2 p.i. *A*, Glucosamine was fed at different doses (10, 50, and 100 mg/mouse/day) starting from day 0 p.i. Mice fed with PBS in parallel served as control. Clinical EAE was scored according to a 0–5 scale and shown ($n = 5$ in each group). *, Comparison between PBS-fed mice and mice fed with glucosamine at 10 mg/day; #, comparison between PBS-fed mice and mice fed with glucosamine at 50 mg/day; @, comparison between PBS-fed mice and mice fed with glucosamine at 100 mg/day; and +, comparison between mice fed with glucosamine at 10 mg/day vs 100 mg/day. *, +, #, and @, $p < 0.05$; **, and ++, $p < 0.01$. *B*, Optimal dose (10 mg/mouse/day) of glucosamine was i.p. injected daily starting from different time points p.i. This dose was chosen based on the results from *A* and a pilot i.p. study (data not shown). *, Comparison between PBS-i.p. and glucosamine-i.p. mice starting from day 0 p.i.; #, comparison between PBS-i.p. and glucosamine-i.p. mice starting from day 13 p.i.; +, comparison between mice receiving glucosamine from day 0 vs day 18 p.i.; and @, comparison between mice receiving glucosamine from day 13 vs day 18 p.i. + and @, $p < 0.05$; ** or ##, $p < 0.01$. *C*, Glucosamine at 10 mg/day or PBS only was administered i.v. starting from day 12 or 18 p.i. * and #, $p < 0.05$. *D*, Glucosamine was administered i.v. at 10 mg/day starting from day 0 p.i. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. One representative experiment of at least two is shown. Gn, Glucosamine.

Glucosamine injection suppressed MOG-induced proliferative response

We studied ex vivo proliferative response to autoantigen MOG35–55 in splenocytes of PBS-i.p. and glucosamine-i.p. mice starting from day 0 p.i. When mice were sacrificed at the acute phase (day 23 p.i.), low proliferative responses were observed in both PBS-i.p. and glucosamine-i.p. mice without Ag stimulation. When cells were stimulated with MOG35–55, a strong proliferative response was observed in PBS-treated mice, and the proliferative response was significantly lower in glucosamine-treated mice ($p < 0.01$; Fig. 4). In contrast, lymphocytes from both PBS- and glucosamine-injected mice responded to Con A at a similar, high level (Fig. 4). No significant difference was found between PBS-treated and glucosamine-treated mice when animals were sacrificed on day 33 (data not shown), at which time the clinical score was similar (Fig. 1*B*).

Glucosamine drives an autoantigen-specific Th2 response in acute EAE ex vivo

To determine the kinetics of Th1/Th2 cytokine production after glucosamine treatment, PBS-i.p. mice and glucosamine-i.p. mice were sacrificed at EAE onset (day 12 p.i.), peak of clinical severity (day 23 p.i.), and chronic phase (day 33 p.i.). Splenocytes were cultured in the presence or absence of autoantigen MOG33–55 peptide for 72 h, and supernatants were assayed for cytokine production. Splenocytes from all groups produced undetectable or low levels of cytokines when these cells were cultured without Ag, which represents spontaneous secretion of cytokines. When stimulated with MOG35–55, splenocytes from glucosamine-treated mice produced significantly, albeit not dramatically, lower levels of Th1/inflammatory cytokines IFN-γ at day 23 p.i. ($p < 0.05$) and IL-17 ($p < 0.05$) at days 12 and 23 p.i. (both $p < 0.05$). A significantly higher level of Th2 cytokine IL-5 was found at both day 12 p.i. ($p < 0.05$) and day 23 p.i. ($p < 0.001$). Significantly higher MOG-induced IL-10 production was also found in glucosamine-treated mice at day 12 p.i. ($p < 0.05$) (Fig. 5). Production of IL-4 at both time points was undetectable. No significant difference for these cytokines between glucosamine-treated and nontreated mice

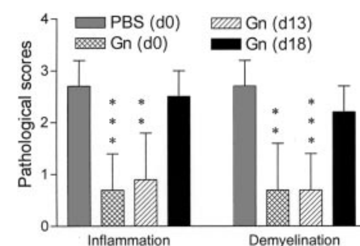

FIGURE 2. Histology of the spinal cords. Mice receiving glucosamine i.p. starting from different time points and PBS from day 0 p.i. were sacrificed on day 23 p.i. ($n = 5$ in each group). Spinal cords were harvested after extensive perfusion, and 5-μm sections were stained with H & E (for inflammation) and Luxol fast blue (for demyelination). Mean values and SD for each group are shown ($n = 5$ in each group). *, Comparison between PBS-treated and glucosamine-treated mice. **, $p < 0.01$, and ***, $p < 0.001$. One representative of three experiments is shown. Gn, Glucosamine.

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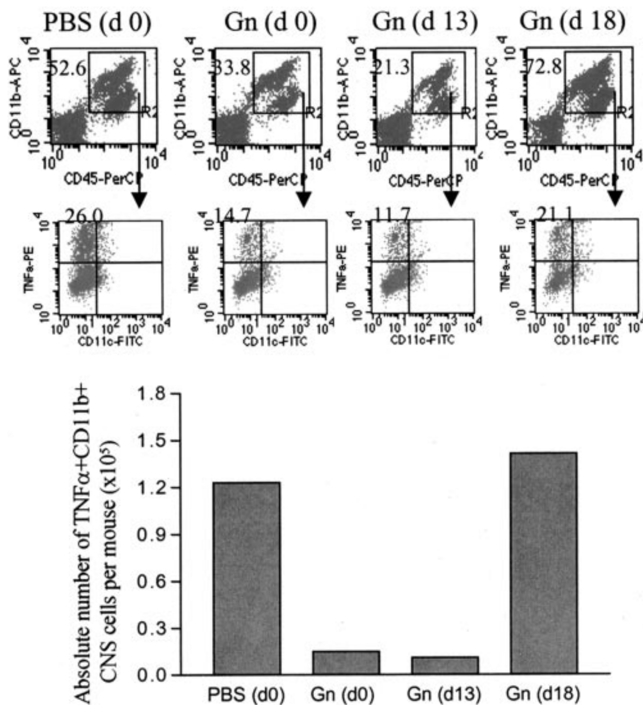

FIGURE 3. Pattern of microglia/infiltrating macrophages and intracellular TNF-α in the CNS. Mice receiving glucosamine i.p. starting from different time points and PBS from day 0 p.i. were sacrificed on day 23 p.i. ($n = 5$ in each group). Spinal cords were harvested after extensive perfusion. MNCs were isolated from pooled tissues, cultured overnight with MOG35–55, and analyzed by flow cytometry. Shown are viable cells (gated based on forward and side scatter parameters). The average numbers of cells per spinal cord were as follows: PBS-i.p. control EAE mice, 9×10^5 cells; glucosamine-i.p. from day 0 p.i., 3.1×10^5; glucosamine-i.p. from day + 13 p.i., 4.4×10^5; glucosamine-i.p. from day + 18 p.i., 8.2×10^5. *Top row*, Pattern of $CD11b^+$ cells. Number indicates the percentage of $CD11b^+$ cells in the gated mononuclear cells. *Middle row*, TNF-α production by $CD11b^+$ (microglia/macrophages) and $CD11c^+$ (CNS DCs) cells. Number indicates the percentage of TNF-α^+ cells in the gated $CD11b^+$ cells. *Bottom row*, Mean absolute number of TNF-α-producing $CD11b^+$ cells in the CNS of each mouse, which was calculated by the total number of MNCs $\times$ percentage of $CD11b^+$ cells (*top row*) $\times$ percentage of TNF-α-producing $CD11b^+$ cells (*middle row*). One representative experiment of three is shown. Gn, Glucosamine.

was found when cells were stimulated with mitogen Con A (data not shown). At the chronic phase (day 33 p.i.), the cytokine profile in glucosamine-treated and PBS-treated mice was similar (data not shown).

Effect of glucosamine on MOG35–55- and Con A-induced proliferation in vitro

When splenocytes of EAE mice were cultured in vitro with glucosamine, the proliferative response to autoantigen MOG35–55 was significantly inhibited at glucosamine concentrations of 1, 10, and 100 μM, with an optimal concentration of 10 μM (Fig. 6*A*). The inhibition was not dose dependent, as no significant inhibition was observed when cells were cultured with a higher concentration of glucosamine (1000 μM). This result also indicated that glucosamine-mediated inhibition was not due to cytotoxicity, as confirmed by LDH assay (data not shown). Glucosamine also inhibited mitogen Con A-induced proliferative response (Fig. 6*B*), indicating that the inhibition by this agent was Ag nonspecific in vitro.

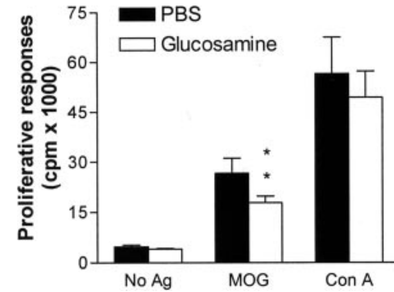

FIGURE 4. Effect of glucosamine on proliferative responses ex vivo. At day 23 p.i., spleens of nontreated EAE mice and mice receiving glucosamine at a dose of 10 mg/day starting from day 0 p.i. were harvested ($n = 5$ in each group). A total of 4×10^5 splenocytes/200 μl was cultured with MOG35–55 at 10 μg/ml, Con A at 5 μg/ml, and without Ag/mitogen for proliferative response. Bars represent mean values $\pm$ SD. Values of p refer to comparison between PBS-i.p. and glucosamine-i.p. groups. **, $p < 0.01$. One representative experiment of three is shown.

Effect of glucosamine on production of cytokines and NO in vitro

We further defined glucosamine-induced Th differentiation of splenocytes of EAE mice in vitro. As shown in Fig. 7, glucosamine significantly suppressed IFN-γ, IL-17, and NO, an inflammatory molecule involved in the development of EAE (17). By contrast, glucosamine significantly up-regulated IL-5 and IL-10. The production of another Th2 cytokine, IL-4, was not detectable (data not shown). These in vitro effects were not Ag specific, as similar results were also observed in cells without stimulation and with

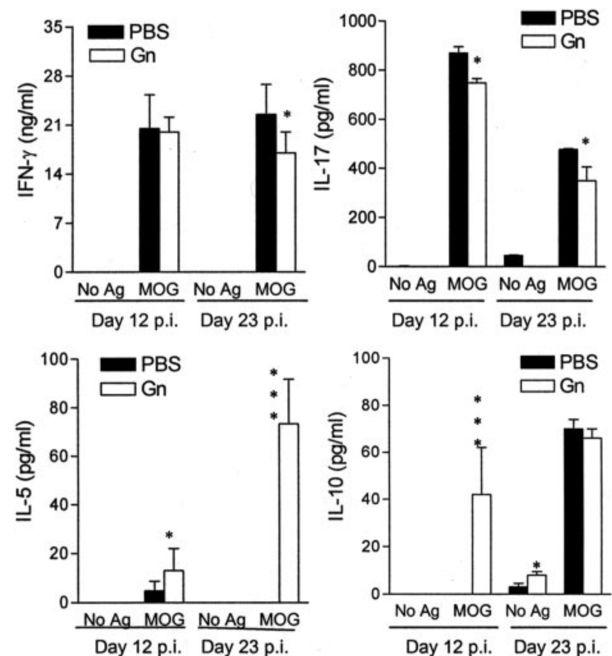

FIGURE 5. Effect of glucosamine on cytokine production ex vivo. At day 12 p.i. (EAE onset), day 23 p.i. (peak), and day 33 p.i. (chronic), spleens of PBS-treated and glucosamine-treated (10 mg/day) mice starting from day 0 p.i. were harvested (n = 5 in each group). A total of 4×10^5 splenocytes/200 μl was cultured with MOG35–55 at 10 μg/ml, Con A at 5 μg/ml, and without Ag/mitogen. Production of cytokines was determined from 72-h culture supernatants by sandwich ELISA. Bars represent mean values $\pm$ SD. Values of p refer to comparison between PBS-i.p. and glucosamine-i.p. groups. *, $p < 0.05$; ***, $p < 0.001$. No difference for these cytokines was observed between treated and nontreated mice at day 33 p.i. (data not shown). One representative experiment of three is shown.

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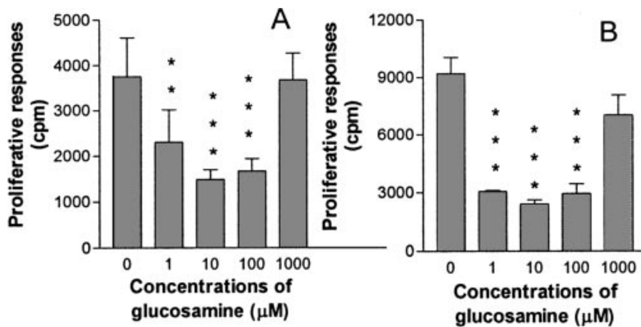

FIGURE 6. Effect of glucosamine on proliferative responses in vitro. Spleens of EAE mice were harvested at day 12 p.i. at the onset of EAE ($n = 5$ in each group). A total of 4×10^5 splenocytes/200 μl was cultured with MOG35–55 at 10 μg/ml (A) and Con A at 5 μg/ml (B) for proliferative response. Glucosamine was diluted in culture medium and added at the various concentrations indicated, maintaining a constant total volume of 200 μl/well. Bars represent mean values ± SD. Values of p refer to comparison between wells with or without glucosamine. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. One representative experiment of three is shown.

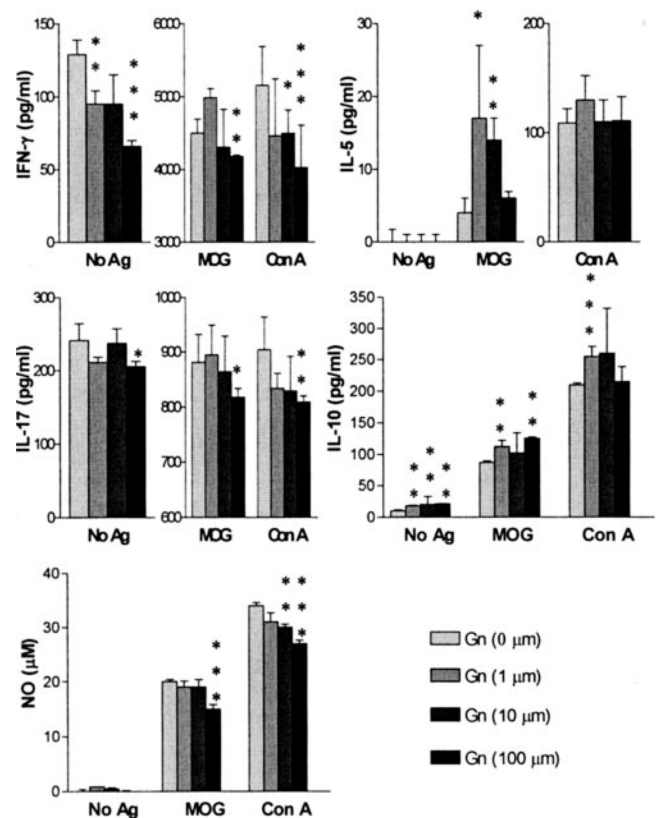

FIGURE 7. Effect of glucosamine on cytokine production in vitro. Spleens of EAE mice were harvested at days 12 p.i. when EAE was at onset ($n = 5$ in each group). A total of 4×10^5 splenocytes/200 μl was cultured with MOG35–55 at 10 μg/ml and Con A at 5 μg/ml. Glucosamine was diluted in culture medium and added at various concentrations indicated, with a constant total volume of 200 μl/well. Production of cytokines was determined from 48-h culture supernatants by sandwich ELISA. Bars represent mean values ± SD. Values of p refer to comparison between wells with or without glucosamine. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. One representative experiment of three is shown. Gn, Glucosamine.

stimulation of Con A. These results, together with the results derived from ex vivo studies (Fig. 7), suggest that glucosamine suppresses EAE by inducing a Th2 response.

Discussion

MS is considered an autoimmune demyelinating disease in the CNS that develops in young adults, affecting >300,000 people in the United States alone (23–25). Despite extensive research to develop effective pharmacological treatments to alleviate exacerbation and chronic neurological damage in MS, current available drugs have limited efficiency and considerable side effects (23–25). Glucosamine, a natural glucose derivative and an essential component of glycoproteins and proteoglycans, has been shown to effectively suppress T cell and APC activation (12, 13) and to prolong graft survival (13), and, more importantly, it has been safely used in humans (8, 9). These results encouraged us to investigate in the present study the effect of glucosamine on EAE, an animal model of MS. We demonstrated that administration of glucosamine by different routes significantly suppressed acute, but not chronic EAE in C57BL/6 mice and reduced the inflammatory infiltration into the CNS. Both ex vivo and in vitro studies showed that glucosamine induced a blockade of Th1 and an up-regulation of Th2 response. These results suggest an effect of glucosamine to induce Th2 response and to inhibit acute autoimmune attack on the CNS.

Activated resident microglia and infiltrating macrophages are considered the main APCs in the CNS in EAE (21, 26, 27). It has been shown that, in the CNS of EAE mice, $CD11b^+$ cells can shift from $CD11c^-$ to $CD11c^+$ (20, 27), indicating that these microglia have differentiated into DC-like cells in situ during CNS inflammation. These DCs express more gene products involved in Ag presentation and become fully competent APC (16, 28). Differentiation into DCs would therefore enhance the APC function of microglia in EAE/MS. In this study, we characterized these populations in the CNS and analyzed their capacity to produce TNF-α, a proinflammatory cytokine involved in the pathogenesis of MS and EAE (3, 4). We found that the degree of inflammation and demyelination in the CNS correlated with the clinical course in glucosamine- and PBS-treated mice. Glucosamine significantly inhibited the percentage and number of activated microglia and infiltrating macrophages in the CNS, and inhibited the capacity of these cells to produce TNF-α. However, only a few $CD11b^+$ cells acquired a DC-like phenotype ($CD11b^+CD11c^+$) in both glucosamine-treated mice and PBS-treated control EAE mice, suggesting a predominant APC function of activated microglia and infiltrating macrophages in EAE. Taken together, our data show that glucosamine administration reduced the infiltration of macrophages, the activation of resident microglia, and the production of inflammatory cytokine(s) in the CNS, resulting in resistance to acute EAE.

The balance of Th1/Th2-type cytokines might have a substantial role in the regulation of autoimmune diseases. MS and EAE have been identified as Th1-mediated autoimmune diseases (1). In contrast, Th2-mediated responses have beneficial effects on the severity and progression of the disease (29, 30), and are one of the major mechanisms underlying Ag-specific immune tolerance induction (31–34). In this study, we demonstrate that glucosamine administered in vivo in EAE mice results in a tendency to shift Th phenotype from a Th1 toward a Th2 type. This is shown by a significant, albeit not dramatic, decrease in levels of IFN-γ and IL-17, but an increase in levels of IL-5 and IL-10. Although the involvement of IFN-γ in the pathogenesis of MS and EAE is controversial, decrease in this cytokine indicates an inhibitory effect of glucosamine on Th1 response. Of note in this study was the ability of glucosamine to suppress the production of IL-17, which has been

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found to play a crucial role in the pathogenesis of EAE (5, 15, 35). The inhibitory effect of glucosamine on IL-17 may be an important factor in the amelioration of EAE. IL-10 and IL-5 are potent Th2 cytokines involved in autoantigen-induced tolerance in EAE (36) and in drug-induced immune suppression in EAE (37) and MS (38). Although IL-4 production was undetectable, probably due to a rapid and pronounced uptake of IL-4 by high affinity IL-4Rs on the cells in the culture (39, 40), the glucosamine-induced increase in IL-5 and IL-10 production ex vivo and in vitro (see below) indicates that glucosamine abrogates EAE not simply as an immunosuppressant, but as an immunomodulator. Administration of glucosamine, thus, provides a novel immunoregulatory approach in autoimmune disorders.

To directly confirm the findings derived from in vivo and ex vivo studies, splenocytes of EAE mice were cultured in the presence or absence of glucosamine. The results show that glucosamine directly inhibits the secretion of inflammatory molecules IFN-γ, IL-17, and NO in vitro in a dose-dependent manner in the 1–100 μM range without affecting cell viability, which is confirmed by similar levels of LDH release (data not shown). A higher dose (1000 μM) of glucosamine is not effective in suppression of T cell proliferation, but is not toxic for these cells. Increased levels of IL-5 and IL-10 induced by glucosamine in vitro further support our ex vivo findings. Of note is that glucosamine regulates both MOG- and Con A-induced responses in vitro. This lack of Ag specificity in vitro may indicate that glucosamine possesses broad immunoregulatory properties, as shown in its suppression of graft rejection (13), CD3-induced T cell activation (12), and other immune responses (10, 11). However, the suppressed MOG-induced, but not Con A-induced, Th1 responses ex vivo (Fig. 5) suggest that glucosamine mainly suppresses T cells at the time of activation. The mechanism underlying this phenomenon remains to be further elucidated.

It has been recently suggested that glucosamine suppresses T cell responses by a direct inhibitory effect on T cell proliferation and by interfering with APC functions (12). Glucosamine also suppresses in vitro DC activation (13). We thus investigated whether the suppression of EAE by glucosamine in the current study is also due to a suppression of APC activation. We did not find differences in costimulatory molecules (B7.1, B7.2, and CD40) or cytokine profile (TNF-α and IL-12) between glucosamine- and PBS-treated EAE mice in peripheral APCs and in in vitro studies (data not shown). However, because NO is mainly produced by APCs (17, 41), inhibition of NO production by in vitro glucosamine in the current study indirectly suggests a suppressive effect of this drug on the function of APCs. Our finding that glucosamine treatment suppressed the production of TNF-α by activated microglia/infiltrating macrophages provides further evidence for the inhibitory effect of glucosamine on activated APCs.

In conclusion, our study demonstrates the in vivo suppressive effect of glucosamine on acute EAE, and provides evidence that glucosamine selectively, although not strongly, suppresses Th1 and induces Th2 response. The ability to delay glucosamine administration until disease onset to ameliorate EAE also fulfills an essential prerequisite for an anti-MS agent, as treatment of patients is started after the onset of MS. The absence of inhibition in the chronic phase of EAE suggests that glucosamine may not be a strong disease-modifying therapy. As this drug effectively suppresses acute EAE, has low or absent toxicity, and has been safely used in humans orally, our study suggests a potential use for this drug alone or in combination with other disease-modifying immunotherapies to enhance their efficacy and reduce their doses in MS and other autoimmune disorders. For example, induction of IL-10 by glucosamine (Figs. 5 and 7) and IFN-β (42–44) might be attained at low doses of each drug. Furthermore, administration of glucosamine provides a novel immunoregulatory approach in autoimmune disorders.

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Disclosures

The authors have no financial conflict of interest.

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