Immunosuppressive effects of glucosamine

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Running title: Glucosamine immunosuppression
Summary:

Glucosamine is a naturally-occurring derivative of glucose, and is an essential component of glycoproteins and proteoglycans, important constituents of many eukaryotic proteins. In cells, glucosamine is produced enzymatically by the amidation of glucose-6-phosphate and can then be further modified by acetylation to result in N-acetylglucosamine. Commercially, glucosamine is sold over the counter to relieve arthritis. While there is evidence in favor of glucosamine’s beneficial effects, the mechanism is unknown. Our data demonstrate that glucosamine suppresses the activation of T-lymphoblasts and dendritic cells in vitro, as well as allogeneic mixed leukocyte reactivity in a dose-dependent manner. There was no inherent cellular toxicity involved in the inhibition and the activity was not reproducible with other amine sugars. More importantly, glucosamine administration prolongs allogeneic cardiac allograft survival in vivo. We conclude that, despite its documented effects on insulin sensitivity, glucosamine possesses immunosuppressive activity and could be beneficial as an immunosuppressive agent.
Introduction

Glucosamine is a naturally-occurring sugar that is synthesised by virtually all cells. Upon uptake, glucose is immediately phosphorylated and enzymatically converted into a series of substrates that will either be converted into glycogen, lipids, proteins or used to generate ATP and CO\textsubscript{2}. 2-3% of glucose-6-phosphate, the immediate intracellular glucose derivative following uptake, is diverted into a pathway known as the hexosamine biosynthesis pathway (1,2). The rate-limiting enzyme, glutamine:fructose-6-phosphate amidotransferase is responsible for the commitment of glucose derivatives into the pathway, ultimately resulting in the formation of glycoprotein precursors (3). Glucosamine is not secreted outside of cells, but exogenously-added glucosamine is taken up by glucose-transporters (GLUT-2 and GLUT-4) and then phosphorylated (4,5).

In 1953, Quastel and Cantero demonstrated that glucosamine possessed tumor-inhibitory activity (6). Since then, a number of reports confirmed the tumoricidal activity of glucosamine (7-16). Glucosamine has been shown to inhibit nucleic acid and protein biosynthesis and irreversible damage to organelles in tumor cells, but not in normal cells (8-15,17-20). In addition,
Glucosamine also inhibits platelet aggregation and ATP release induced by Staphylococcus aureus, ADP, epinephrine and collagen (21). The mechanisms by which glucosamine acts are not completely clear, however, it has been shown to alter the ultrastructure of plasma and intracellular membranes (9,22), to inhibit membrane transport of nucleosides (14,15) and reportedly to shift its distribution from glycoproteins to glycolipids (22).

O’Neill et al. demonstrated that monosaccharides, especially amino-sugars, were able to inhibit cytotoxic T-lymphocyte function in culture preventing target lysis in a CTL clone-specific manner (23). In 1989, Yagita et al. further demonstrated that free hexosamines were able to inhibit NK cell cytotoxicity in culture (24,25) and that hexosamine release by tumors could, in part, explain escape of tumors from immune cell lysis (24). Since then, other than the observation that Amiprilose, a synthetic monosaccharide, was able to attenuate T-cell activation in a dose-specific manner (26), no other work has been published on the utility of sugar derivatives, especially amino sugar derivatives (glucosamine, mannosamine, lactosamine, fructosamine), as immunoregulatory agents. However, a recent report by Gouze et al. demonstrated glucosamine-dependent inhibition of NF-κB activity in rat chondrocytes and IL-1β bioactivity by upregulation of the type II IL-1 decoy receptor (27).

Glucosamine has received considerable attention in the past five years as an agent that may be beneficial for arthritis in a number of studies (28-31). Sold
mainly over the counter in various formulations (glucosamine sulfate, glucosamine sulfate with chondroitin sulfate), the manufacturers suggest that the beneficial effects of their compounds are due to the reconstruction of joint cartilage, of which one of the constituents is glucosamine in the form of glycoproteins of structural proteoglycans. A recent study in humans demonstrated beneficial effects of glucosamine in arthritis, although no firm conclusions could be made (28). The actual mechanism by which glucosamine may benefit the patient remains unknown, although very recent investigation suggests that it may interfere with pro-inflammatory cytokine action on human chondrocytes (32).

Glucosamine, however, induces insulin resistance in the absence of high glucose or glutamine (2) as well as insulin resistance in isolated rat muscle (33). Glucosamine has also been shown to modulate the effects of insulin and glucose on pyruvate kinase (34), glycogen synthase (33,34) and transforming growth factor alpha gene expression (35). A short-term exposure of cultured rat adipocytes to glucosamine decreases GLUT-4 activity and longer, 16 hour incubations, result in decreased GLUT-4 cell surface levels (36). Furthermore, glucosamine infusion can induce insulin resistance in normoglycemic, but not hyperglycemic rats and this is accompanied by impaired GLUT-4 translocation to the cell surface of skeletal muscle in response to insulin (37). Insulin sensitivity in rat cardiac muscle and liver has also been shown to be affected by
glucosamine infusion (38). More importantly, acute glucosamine infusion into humans has been demonstrated to mimic some of the metabolic aspects of insulin resistance in human type 2 diabetes mellitus (39).

To unravel the mechanisms involved in the possible beneficial effects of glucosamine, despite its reported effects on insulin sensitivity, we have begun to examine the effects of glucosamine on immune cell activation and inflammatory processes. In initial studies, we have discovered that glucosamine addition to immune cells in vitro prevented both their activation, and their ability to initiate mixed leukocyte reaction. More importantly, a single intravenous injection of glucosamine daily was able to prolong cardiac allograft survival in mice. Based on these data, we suggest that glucosamine alone or in its different formulations could be considered as a novel immunosuppressive agent with potential clinical utility.
Experimental Procedures

Generation of Reporter T-cell Lines:

The Jurkat T-lymphoblast cell line used in this study is a human T-lymphocyte precursor cell line that responds identically to non-specific, non-antigenic stimulation signals and available from ATCC (ATCC TIB-152) (40,41). It has been extensively used to identify and unravel T-cell activation and signaling pathways (42-53). To test the effects on T-cell activation of a number of agents, we designed appropriate Jurkat-based reporter cell lines. We engineered the Jurkat cell line to stably express the beta galactosidase gene under the control of four tandemly-arranged NFAT cis-acting enhancer elements derived from the NFAT-PathDetect reporter plasmid (Stratagene, La Jolla, CA). NFAT (nuclear factor for the activation of T-cells) describes the family of intracellular transcription factors that are among the very first to be activated in response to T-cell antigen receptor ligation, or non-specific signals (54-58). NFAT quickly translocates to the nucleus and activates the IL-2 gene, one of a number of genes whose products is important for maintaining and propagating the T-cell activation signal. The engineered Jurkat T-cells which we term JLZB (beta galactosidase reporter), express beta galactosidase when exposed to factors that engage their T-cell receptors in a specific manner or by chemical, non-specific factors like phorbol-13-myristate acetate (PMA) and ionomycin. Cultures were
exposed to hygromycin (Gibco-BRL, Gaithersburg, MD) to select stably-transfected clones. Individual clones were isolated and tested for NFAT inducibility. In a similar fashion, we also created a stable Jurkat cell line constitutively expressing the green fluorescence protein (GFP) under the control of the SRalpha promoter (consisting of the SV40 early promoter and the R-U5 segment of the human T-cell leukemia virus type 1 LTR). We designated this cell line as JSR-GFP. Stably-transfected JSR-GFP clones were selected with 0.25 mg/mL Zeocin (InVivoGen, San Diego, CA). To determine promoter activation, we cultured 3 x 10^6 cells of each clone selected overnight in 1 mL in ionomycin (1 microgram/mL; Calbiochem, La Jolla, CA) and PMA (25 nanograms/mL; Sigma, St-Louis, MO) in RPMI 1640, 10% fetal bovine serum and antibiotics (Gibco-BRL, Gaithersburg, MD) and the next day we quantitated the amount of beta-galactosidase by a commercially-available kit (Tropix Systems, Bedford, MA) or by FACS analysis of JSR-GFP clones. We selected the clones exhibiting the highest inducibility for further experimentation.

Effects of Glucosamine on Reporter Cell Activation

To examine the effects of glucosamine on T-cell activation we added pure glucosamine (Sigma, St-Louis, MO) dissolved in PBS (Sigma, St-Louis, MO) to cultures of JLZB or JSR-GFP cells in RPMI 1640, 10% fetal bovine serum and antibiotics. Glucosamine was added to final concentrations ranging from 1
micromolar to 10 mM for a period of 18-24 hours in the presence or absence of PMA and ionomycin at activating concentrations. As control, we incubated parallel cultures in equal concentrations of galactosamine and mannosamine (all purchased from Sigma, St-Louis, MO). Cells were collected, centrifuged at 600 x g and the pellets washed extensively in PBS. The cells were subsequently lysed in reporter gene lysis buffer (Promega, Madison, WI) or directly analysed by FACS (JSR-GFP) in a FACS Vantage SE flow cytometer (BD Instruments, Palo Alto, CA). Reporter cell activation was determined by assessing the level of beta galactosidase activity in the lysates of JLZB cells (Tropix Systems, Bedford, MA). Concurrently, secreted IL-2 levels were determined in the supernatant of the cells by a commercially-available ELISA (R&D Systems, Minneapolis, MN). Cell viability in all instances and following treatment was measured using a commercially-available reagent based on the MTS assay (CellTiter96; Promega, Madison, WI).

To examine the reversibility of the effects of glucosamine on reporter gene activity, JLZB cells were first stimulated with PMA and ionomycin in the presence or absence of 10 mM glucosamine. 24 hours later, the cells were washed, media were replaced and the cells were once again stimulated with PMA and ionomycin. Beta galactosidase activity was determined as described earlier.

Measurement of ATP levels in glucosamine-treated Jurkat T-cells
To determine the effects of glucosamine administration on ATP levels in cultured Jurkat T-cells, we measured ATP indirectly using a commercially-available reagent (ENLITEN, Promega, Madison, WI). The assay is based on the ATP-dependent conversion of luciferin to oxyluciferin, AMP, pyrophosphate, carbon dioxide and light, measured in a luminometer (560 nm). The intensity of emitted light is directly proportional to the amount of ATP present in the sample. Jurkat T-cells were stimulated with PMA and ionomycin as described earlier in the presence or absence of 10 mM glucosamine for 18 hours. The cells were extensively washed and equal numbers of cells from each treatment group as well as untreated cells were then lysed. The lysate was cleared by centrifugation and an aliquot of the supernatant was used to assess ATP levels using the ENLITEN reagent.

Comparison of the effects of glucosamine to cyclosporin A and tacrolimus on reporter cell activation

The effects of glucosamine were compared to those of immunosppressive agents in clinical use (cyclosporin A and tacrolimus (FK-506)) and examined using the JLZB reporter cell line. Following the addition of glucosamine, galactosamine or mannosamine (at different final concentrations ranging from 10 nM to 10 mM final), cyclosporin A (Sandimmune, 100 nanograms/mL; Novartis, Basel, Switzerland) or tacrolimus (10 nanograms/mL; Prograf, Fujisawa,
Deerfield, IL) to 3 x 10^6 cells, PMA and ionomycin were added and the cells incubated for a period of 18-24 hours. The next day, the culture supernatants were collected and the cells lysed. Beta galactosidase activity was determined as described earlier.

Effects of Glucosamine on Dendritic Cell Activation

Five-eight week-old mice were obtained from the Jackson Laboratories (Bar Harbor, ME). C57/BL10 (B10) mouse bone marrow-derived dendritic cells (DC) were obtained from bone marrow progenitors cultured with GM-CSF and IL-4 (R&D Systems, Minneapolis, MN) essentially as described (59,60). By this method the purity of DC is usually >85% as assessed by FACS analysis for DC cell surface markers (CD86, CD80, CD40, DEC-205, class I and class II MHC (purchased from BD Pharmingen, Palo Alto, CA) (59,60). 1 x10^5 DC were treated with glucosamine, galactosamine, mannosamine (all at 10 mM final) or PBS for 18-24 hours and then stimulated with 25 micrograms/mL lipopolysaccharide (LPS; Sigma, St-Louis, MO)) for another 18-24 hours. At the end of the stimulation, the culture supernatants were assessed for nitrite production, a marker of DC activation, using the Griess reagent method (Promega, Madison, WI).
Effects of glucosamine on allogeneic mixed MLR in culture

Co-cultures of irradiated B10 splenocytes and C3H/HeJ (C3H; Jackson Laboratories, Bar Harbor, ME) mouse T-lymphocytes were cultured in the presence or absence of glucosamine, galactosamine, mannosamine (2.5, 5, 10 mM final concentration) or media alone for a period of 5 days. At the end of the co-culture, tritiated thymidine was added for 18 hours and the radioactivity incorporated into the cells was determined by liquid scintillation counting.

Effects of glucosamine on cardiac allograft survival

To examine the effects of glucosamine on cardiac allograft survival, heterotopic transplantation of C3H hearts into B10 mice was performed as described (59). There is complete mismatch at the MHC between these two species. Each recipient was conditioned with one injection of 8, 20 or 40 micromoles glucosamine i.v one day prior to transplantation and one daily injection i.v. of 40 micromoles glucosamine beginning on the second day following transplantation until the end of the experiment (defined as the time of transplant rejection). To compare the efficacy of glucosamine with immunosuppressive agents in clinical use, we also treated a subset of mice with FK-506 (0.5 mg/kg/day) or cyclosporin A (10 mg/kg/day) once a day for seven days following transplantation alone or in combination with different amounts of
glucosamine. As control we also gave a single daily injection of 40 micromoles i.v. of mannosamine or galactosamine in parallel sets of mice. Rejection of the transplant was determined to be the time at which palpable beating of the heart ceased.
Results

Effects of Glucosamine on T-cell and dendritic cell activation

The effects of glucosamine on immune cell activation in vitro were first examined. Three parameters were evaluated: T-cell activation, dendritic cell activation and allogeneic mixed leukocyte reaction. To assess T-cell activation, we added glucosamine to Jurkat T-lymphoblasts stably transfected with the beta galactosidase gene under the control of cis-acting NFAT enhancer elements in tandem (JLZB cell line). In Figure 1A we demonstrate that the addition of 10 mM glucosamine to JLZB reporter cells, for a period of 18 hours in the presence of chemical activators of T-cell function (PMA and ionomycin), resulted in a nearly complete prevention of expression of beta galactosidase as assessed by beta galactosidase activity. Additionally, in Figure 1B, we show a significant suppression of IL-2 production by the same cells in the culture supernatants. To compare the efficacy of glucosamine to commonly-used immunosuppressive agents, we assessed T-cell activation in the JLZB reporter cell line in the presence of cyclosporin A or tacrolimus (FK-506). In Figure 1A we show that glucosamine at concentrations as low as 500 micromolar is as effective as clinical doses of cyclosporin (100 ng/mL) or FK-506 (10 ng/mL) in suppressing the activation of the JLZB reporter cell line. The results also demonstrate that
glucosamine inhibits NFAT-dependent transcription stimulated by PMA and ionomycin at levels similar to those of cyclosporin.

To control for potential non-specific effects by glucosamine we examined the ability of other aminated sugars, galactosamine and mannosamine, on IL-2 production by Jurkat T-cells in response to PMA and ionomycin. Figure 1C demonstrates that galactosamine (Gl) was unable to prevent IL-2 production in response to a PMA/ionomycin challenge. Interestingly, mannosamine was able to achieve significant prevention of IL-2 production (Mn) but not at levels achievable by glucosamine (100 +/- 20 ng/mL IL-2 in Jurkat cell supernatants vs. 50 +/-36 ng/mL , in mannosamine and glucosamine-treated cells, respectively, following PMA/ionomycin stimulation). To rule out the possibility that glucosamine exerted a non-specific toxic effect on Jurkat T-cells, we examined cell viability based on MTS assessment using a commercially-available reagent (CellTiter 96® Aqueous, Promega). This assay determines metabolically-viable cells which converts the MTS substrate into an aqueous soluble formazan by dehydrogenases found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. Figure 1D shows that no significant differences were observed in the viability of wild-type and JLZB cells treated or untreated with glucosamine. The observable decrease in viability of PMA/ionomycin-treated cells can be attributable to activation-induced cell death, very likely mediated through Fas ligand-Fas interactions, since one mechanism
by which the response of activated T-cells is terminated is by fratricidal Fas-Fas
ligand-induced apoptosis.

To rule out the possibility that glucosamine induces irreversible changes in
the response of JLZB cells to PMA/ionomycin, we first cultured JLZB cells
overnight in the presence of 10 mM glucosamine. The cells were then washed
twice in PBS and fresh medium was added. PMA and ionomycin were added 24
hours thereafter. In Figure 1E, we show that these JLZB cells responded
appropriately to PMA/ionomycin demonstrating that the effects of glucosamine
are reversible. Finally, to show that glucosamine-induced suppression of NFAT
promoter element-driven LacZ reporter expression was specific for NFAT-
dependent transactivation and not a non-specific effect on general promoter
activity, we tested the effects of glucosamine on JSR-GFP cells, stably-
transfected Jurkat cells with the constitutive SRalpha promoter driving the
expression of a GFP cassette. In Figure 1F, we present data from FACS analysis
of glucosamine-treated and untreated cells in the form of mean fluorescence
intensity. We demonstrate GFP expression in JSR-GFP cells treated with
glucosamine following PMA/ionomycin stimulation. The fluorescence profiles
between glucosamine-free cell cultures and glucosamine-treated cultures
following PMA/ionomycin stimulation was identical as illustrated in Figure 1F. It is
worth noting that in prior experiments whose data are illustrated above (Figure
1A) beta galactosidase gene expression under the control of NFAT elements is
suppressed by glucosamine in contrast with what is observed in the same cell
background where a reporter gene is driven by a “constitutive” promoter (SRalpha). To determine if glucosamine affected the levels of ATP, we measured luciferin conversion into light in Jurkat T-cells cultured in 10 mM glucosamine overnight with or without PMA/ionomycin activation. Figure 1G demonstrates that ATP levels in the supernatants of glucosamine-treated cells (10 mM final concentration) with or without PMA/ionomycin stimulation were no different than those what were not exposed to the sugar. Finally, another indication of normal metabolic activity of glucosamine-treated cells was that constitutive expression of GFP in JSR-GFP cells and inducible LacZ expression in JLZB cells was easily inhibited by cycloheximide but not by 10 mM glucosamine (data not shown).

Dendritic cells are the most potent immunostimulatory cells yet characterized. They are at the center of a complex immune cell network and define, if not dictate the nature of the immune response and its strength. Dendritic cells can either traffic through tissues or can exist within tissues as resident cells. Upon local disruption of tissue integrity by foreign pathogen invasion or by endogenous changes, dendritic cells will acquire molecules by a variety of uptake pathways and will migrate to the local lymphoid organs. There, they will engage naïve T-cells via class I and class II MHC-TCR interactions and activate the proliferation of the engaged T-cell (61,62). In culture, dendritic cell activation can be ascertained by the levels of lipopolysaccharide (LPS)-induced nitrite production reflecting the activation of the inducible nitric oxide synthase gene through the NF-kappaB pathway (63,64). Figure 2 shows the nitrite output
of DC treated with LPS in the presence or absence of 10 mM glucosamine for an 18 hour incubation. Nitrite levels are significantly reduced in DC treated with LPS in the presence of glucosamine.

Another indicator of dendritic cell function is the mixed leukocyte reaction where T-cell responder proliferation is measured as an index of the number of stimulator antigen presenting cells. We examined the effect of glucosamine addition in an allogeneic mixed leukocyte reaction (MLR). Figure 3 demonstrates a dose-dependent glucosamine suppression of allogeneic MLR. The addition of 10 mM glucosamine at the outset of the co-culture completely prevented the allogeneic MLR while lower doses achieved partial, yet significant suppression.

Effects of glucosamine on cardiac allograft survival

Finally, we examined whether glucosamine treatment could facilitate allograft survival. Table I illustrates the median survival time of allogeneic heart allografts in mice treated with 40 millimoles glucosamine i.v one day prior to transplantation and one daily injection i.v. of 40 millimoles glucosamine beginning on the second day following transplantation until the end of the experiment. The same table compares the efficacy of daily glucosamine injections and a seven-day treatment of conventionally-used immunosuppressive agents (FK-506 and cyclosporin A). Allograft survival was significantly increased in the glucosamine-treated mice compared to untreated controls (MST=18.7 vs.
10.0 days, p<0.05). Glucosamine also appears to be efficaceous compared with standard pharmacologic agents in current clinical use.
Discussion

We have shown that glucosamine is an efficacious agent that can suppress the activation of T-cells and dendritic cells, two crucial cells involved in immune responses. The suppressive effect is specific and reversible and cannot be attributable to cell death. Furthermore, the effect is most potently achieved by glucosamine in comparison to other amine sugars. Galactosamine had no effect and mannosamine, although able to achieve a significant effect, was not able to achieve suppression levels comparable to those of glucosamine. Other sugars tested (glucose, mannose, fructose, fructosamine, lactosamine) were unable to prevent PMA/ionomycin-induced activation of Jurkat or JLZB cells as assessed by beta galactosidase activity in JLZB lysates or IL-2 production (data not shown). Finally, the lack of an effect of glucosamine on a “constitutive” promoter (SRalpha) compared with NFAT reporter elements demonstrates that glucosamine acts by interfering with specific, though as-yet unidentified, molecular signaling pathways. While a number of studies in tumor cell culture and adipocytes demonstrate that glucosamine can affect the ATP and uridine pools, our data demonstrate that, in Jurkat T-cells, ATP levels were not affected at the maximal concentration of glucosamine used (10 mM) (14,15,17-20,22,65). We were unable, however, to reliably measure uridine levels or uridine-
glucosamine metabolites even by HPLC analysis (data not shown). Although we do not believe that depletion of uridine pools accounts for the glucosamine effects we observed, a more formal examination in the context of mechanistic studies of the effects of glucosamine is warranted. Concurrently, we do not believe that the responses we observed in the MLR were due to impotent counter-receptor interactions (i.e., MHC: T-cell receptor; B7:CD28) consequent to glucosamine-modulated underglycosylation of immunostimulatory molecules at the surface of the dendritic and T-cells in co-culture, as shown in a previous study (66).

We suggest that glucosamine contributes to, or is itself a direct substrate of glycosyltransferases involved in post-translational modifications of signal transduction pathways which in T-cells involve the NFAT family of transcription factors. Post-translational modification of proteins, especially transcription factors, by O-linked glycosylation is plausible as a means of regulating cell growth and function. A number of studies have demonstrated that O-linked glycosylation is an important post-translational modification of proteins and may affect protein function to the same degree as protein phosphorylation (reviewed in (67-69)). Proteins as diverse as nucleoporins, RNA Polymerase II, SP1, estrogen receptor, eIF-2, cytoskeletal proteins, p53 and nitric oxide synthase are O-glycosylated and their function is altered depending on state of glycosylation (reviewed in (67-69) and (70)). Our data demonstrate that NFAT-dependent reporter gene expression is affected by glucosamine and raise the possibility that
NFAT transcription factors and/or other proteins that culminate in NFAT translocation into the nucleus, may be targets of O-glycosylation which negatively regulates their activity.

Another potential mechanism of glucosamine actions could affect the oxidation state of cells. Kaneto et al. demonstrated a glucosamine-dependent enhancement of hydrogen peroxide levels in pancreatic beta cells and concluded that activation of the hexosamine pathway leads to deterioration of beta-cell function through the induction of oxidative stress rather than O-linked glycosylation. They also observed a significant decrease in DNA binding activity of PDX-1, an important beta cell transcription factor (71). Our data, however, indicate that oxidative stress is highly unlikely to account for the suppression of the T-cell and dendritic cell activation we observed. Nonetheless, mechanisms suggested by the data of Kaneto et al. are currently under investigation.

Our results also indicate that glucosamine is an effective agent in prolonging allogeneic cardiac allograft survival in mice. One could potentially extrapolate from these data to partly explain the reported beneficial effects of glucosamine in arthritis. By suppressing immune activity, synovial tissue could regenerate unimpeded. The absence of soluble mediators of inflammation and pain generated by immune cells at the site of cartilage erosion could provide the patient with an increased sense of well-being that may or may not result in any remarkable long-term therapeutic effect. In over-the-counter formulations, glucosamine dosage in capsular form does not usually surpass 1.5 grams per
day. Therefore, glucosamine ingested in capsule form may not result in critical tissue levels required to achieve optimal therapeutic effect. This may be due to very quick clearance following oral ingestion, short half-life and/or sub-optimal dose. Carefully-controlled studies evaluating clearance, half-life and maximal safe dosage to achieve measurable anti-inflammatory and immunosuppressive effects will very likely yield answers on the potential to translate our findings to humans.

Glucosamine, however, has been demonstrated to induce insulin resistance in a variety of models including in humans (39) (36-38,72-75) and therefore, any anti-inflammatory or immunosuppressive benefit will have to be carefully considered with this in mind. It is noteworthy, however, that in humans, continuous infusion of glucosamine was necessary to achieve a state of insulin resistance (39). It is possible that transient glucosamine exposure could achieve immunosuppression without inducing or promoting insulin resistance. This possibility requires careful examination.

One other point that our data raise is the possibility that users of glucosamine may be at risk for subtle compromises of immune responses. Whether this is a real concern remains to be determined, but the widespread availability of this compound warrants carefully-controlled studies to assess any potential risk that long-term use may confer on immune activity in humans.

Finally, if our data can be clinically translated, it may be possible to use glucosamine formulations alone or in conjunction with conventional immunosuppressive agents at doses much lower than in current clinical use to
achieve long-term transplant survival with a significantly decreased risk of the
toxicity that is often associated with conventional pharmacological agents.
Mechanistically, its mode of action in immune cells remains to be determined and
is under active investigation in our laboratory.
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Table I: Heart allograft survival

| Treatment          | Allograft survival (individual hosts) in days | MST (days) |
|--------------------|-----------------------------------------------|------------|
| None               | 7, 8, 9, 14, 12, 6, 7, 8, 10, 12              | 10         |
| FK-506             | 22, 15, 18, 19, 24, 25,                       | 21         |
| Cyclosporin A      | 19, 18, 17, 18, 19, 20, 19                    | 19         |
| Glucosamine        | 17, 20, 15, 15, 20, 21, 23                    | 19         |
| Galactosamine      | 9, 8, 12, 7                                   | 9          |
| Mannosamine        | 7, 6, 7, 8                                    | 7          |
| FK-506+Glucosamine| 33, 28, 43, 10, 29, 15, 17, 24, 21, 8         | 26         |

P<0.0001 (2-way ANOVA): Glucosamine compared with no treatment.

Table I. Median survival time of cardiac allografts in mice treated with glucosamine. Comparison is made among the survival of allografts in mice treated with glucosamine, cyclosporin A or FK-506. Data are shown as median survival times. The differences are statistically-significant as assessed by log-rank analysis (P<0.05) and by 2-way ANOVA.
A. **Glucosamine prevents NFAT-driven beta galactosidase expression in JLZB cells.** Beta galactosidase expression is significantly suppressed in JLZB cells treated with glucosamine and exposed to activating concentrations of PMA and ionomycin for 18-24 hours. The bars represent the beta galactosidase activity (shown as relative light units; RLU) in JLZB cells cultured in 10 mM glucosamine (Gn), cyclosporin A (CsA) or FK-506 (FK) for a period of 18-24 hours and then stimulated for an additional 18-24 hours with activating concentrations of PMA and ionomycin (P+I). The last three bars show the beta galactosidase activity of JLZB cells treated with PMA and ionomycin in the presence of different
concentrations of glucosamine. The results shown are representative of three independent experiments in triplicate +/- standard error of the mean (S.E.M). Ctrl: control, untreated cells; P: (PMA=phorbol-13-myristate acetate); I: ionomycin; Gn: Glucosamine at 10 mM, CsA; cyclosporin A; FK: FK-506.

B. Glucosamine suppression of IL-2 production. JLZB cells were treated with glucosamine and activating concentrations of PMA and ionomycin. 18-24 hours later, IL-2 levels were measured by ELISA. The bars represent detectable IL-2 from triplicate determinations and the error bars the S.E.M. P: phorbol-13-myristate acetate; I: ionomycin; Gn: Glucosamine at 10 mM. IL-2 levels were below the limit of assay detection where no bars are present.
C. **Comparison of glucosamine with other amine sugars in suppressing IL-2 production in T-cells.** Galactosamine and mannosamine were added to wild-type Jurkat cells at a final concentration of 10 mM. After an overnight culture, PMA/ionomycin was added and IL-2 levels were assessed by ELISA in the supernatants the day after. Bars represent the amount of secreted IL-2 in pg/mL and the error bars represent the standard error of the mean. Results shown are representative of three separate experiments in triplicate. Gn: glucosamine (10 mM); Mn: mannosamine; Gl: galactosamine; P: PMA; I: ionomycin.
D. Effect of glucosamine on metabolic viability of T-cells in culture. Cell viability was assessed in cultures treated with glucosamine and in parallel cultures further exposed to PMA/ionomycin. The OD$_{490}$ is directly proportional to the metabolically viable cell number. Results are representative of two separate experiments in triplicate where the error bars indicate the standard error of the mean. WT Jurkat: wild-type Jurkat cells; JLZB: Jurkat cells stably-transfected expressing LacZ under the control of NFAT promoter elements.
E. Reversibility of the effect of glucosamine on T-cells in culture. NFAT reporter activity is shown (in RLU-relative light units) in JLZB cell lysates. Cells were previously treated with glucosamine (10 mM), extensively washed with PBS, cultured overnight in normal growth medium and then stimulated with PMA/ionomycin. The error bars represent the standard error of the mean. Data shown are from two separate experiments in triplicate.
F. Glucosamine does not suppress gene expression from a constitutive gene promoter. Glucosamine was added to JSR-GFP cells followed by PMA/ionomycin stimulation. Fluorescence levels were quantified by FACS. The bars represent the mean fluorescence intensity of gated cells that excluded dead cells (propidium iodide-positive). The FACS profiles are also shown on the right-hand side. P: phorbol-13-myristate acetate; I: ionomycin; Gn: Glucosamine at 10 mM. Each bar represents the pooled average of triplicate cultures.
G. Glucosamine does not significantly alter ATP levels in Jurkat T-cells in culture. Glucosamine-treated Jurkat T-cells were stimulated with PMA and ionomycin (P+I) and 18 hours later, the cleared lysates were assayed for ATP levels by an indirect luminometric assay. The bars represent the mean of the relative light intensity (RLU) measured in a microplate luminometer (triplicate determinations). The error bars indicate the standard error of the mean.
Figure 2

Glucosamine suppression of dendritic cell activation. Addition of LPS for 18-24 hours in bone marrow-derived dendritic cell cultures treated with glucosamine did not result in nitrite production at levels detected in cultures from untreated, LPS-treated dendritic cells. LPS: lipopolysaccharide; Gn: glucosamine.
Figure 3

Glucosamine suppression of allogeneic mixed leukocyte reactivity. Co-culture of irradiated bone marrow-derived dendritic cells with purified allogeneic T-cells in the presence of glucosamine results in a dose-dependent suppression of T-cell proliferation. Stimulator cell number remained constant while the responder cell number varied as shown on the x-axis. The results shown are representative of three independent experiments. C.p.m.: counts per minute
Immunosuppressive effects of glucosamine
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J. Biol. Chem. published online August 9, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204924200

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