Effect of Gliotoxin on Development of Diabetes Mellitus in Diabetes-Prone BB/Wor Rats

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Abstract. The object of this investigation was to determine if gliotoxin, an immunomodulating fungal secondary metabolite, is capable of preventing the development of autoimmune diabetes mellitus in diabetes-prone BB/Wor rats. Chronic treatment, consisting of 1 μg gliotoxin/g of body wt administered three times weekly from the age of 30 days through 120 days, reduced the incidence of diabetes from 90% diabetic by 120 days among vehicle-treated animals to 56% diabetic among gliotoxin-treated animals. This result was significant by life table analysis. Animals treated with gliotoxin maintained lower serum glucose levels even in the pre-diabetic state than control (vehicle-treated) rats. Gliotoxin at levels used in this study showed no appreciable effect on the viability of rat insulinoma (RIN 38) cells in culture and only slightly decreased their insulin secretion. Animals chronically treated with gliotoxin showed weight gains comparable to those seen in controls, and the effect of gliotoxin on peripheral blood leukocyte counts was not significant. The possibility that gliotoxin exerted its effect through immunomodulating effects was implied by the loss of white pulp in splenic follicles of gliotoxin-treated animals.

Keywords: Type I diabetes, BB rat, gliotoxin, immunomodulation, autoimmunity, diabetes prevention

Introduction

Type I diabetes is recognized to be essentially an autoimmune phenomenon, although the precise details of immune defect or dysregulation involved in its pathogenesis, either in humans or animal models, have not been fully clarified. However, because of the autoimmune nature of the condition, immunosuppressive drugs such as cyclosporin A have been found to alter the course of the disease in animal hosts [1-3].

In recent years, the ability to identify individuals who are at the earliest stages of type I diabetes development [4] has allowed clinical testing of immunosuppressive regimens that delay full-blown disease with some success in young subjects [5]. Work in our laboratory has involved a fungal secondary metabolite with immunomodulating activity which we were interested in evaluating as an alternative to cyclosporin A. This fungal product, gliotoxin, is an epipolythiodioxopiperazine and is chemically distinct from the macrocyclic drug, cyclosporin A [6,7]. Gliotoxin may therefore have distinct effects on the immune system, in contrast to those of currently available immunosuppressive drugs.

In this study we examine the effect of gliotoxin on the development of autoimmune diabetes in BB/Wor rats and present preliminary evidence of its apparent lack of significant toxicity at the dosages used in this investigation.

Materials and Methods

Gliotoxin (mw 324) was purchased from Sigma Chemical Company (St. Louis, MO). It was obtained as a purified compound in sealed vials. Initial solvation was in HPLC grade methanol because of limited aqueous solubility. Subsequent dilutions were made...
in sterile glycerol for animal injection or in RPMI-1640 for use with cell culture.

To evaluate the potential toxicity of gliotoxin on pancreatic cells, RIN 1046-38 (RIN 38) insulinoma cells were exposed to gliotoxin and their viability determined by trypan blue dye exclusion. Insulin release into the culture medium was measured by radioimmunoassay. RIN 38 cells were grown 48 hr in RPMI-1640 medium with 5% fetal bovine serum and 2.8 mM glucose at 37°C with 5% CO₂. At the end of this incubation, the cells were washed with Krebs-Ringer’s Buffer (KRB). To 1 ml of KRB, glucose was added to a final concentration of 2.8 mM for 1 hr (37°C, 5% CO₂). This medium was subsequently removed, cells were then washed gently with KRB, and 1 ml of KRB containing 2.8 or 16.7 mM glucose and 0, 0.5, 1, or 5 μg gliotoxin/ml was added. Cells were incubated 1 hr and the supernatant removed and frozen for RIA. The RIN 38 cells were mixed with trypan blue and counted in a hemocytometer. Viable cells were considered to be those that were unstained.

RIA was preceded by iodinating insulin which involved combining 10 μl of insulin (0.4 μg/ml), 20 μl 0.25 M phosphate buffer pH 7.5, 14.2 mCi ¹²⁵I, and 15 μl chloramine. After 45 sec, 50 μl 0.25 M phosphate buffer were added. The mixture was passed over a carboxymethylcellulose column and eluted with 0.05 M phosphate buffer. The unreacted iodine and labeled fractions were identified by gamma counter.

Guinea pig anti-porcine insulin, which crossreacts with rat insulin, was used at a 1/500,000 dilution. A seven point standard curve (0-11.25 ng insulin/ml) was prepared. The samples were combined with the antibody for four hr at room temperature. Radiolabeled insulin was then added to each tube and the tubes were incubated overnight. Dextran-coated charcoal (2 ml, 25 mg/ml) was added to each tube to remove unbound insulin and the mixture was centrifuged at 500 x g at 8°C. The supernatant fluid was counted in a gamma counter (12 min per tube) set for ¹²⁵I. Radioactivity of each unknown was compared to the standard curve to determine insulin concentration. All samples were tested in duplicate and results were reported as the mean.

Use of experimental animals was under the jurisdiction of the local institutional animal care and use committee, which approved the work presented in this paper. The animal facility is AAALAC accredited and the animal care personnel were specially trained in the care of BB/Wor rats. The appropriate care and handling of BB/Wor rats has been described elsewhere [8,9].

BB/Wor rats were obtained from the University of Massachusetts breeding colony and equal numbers of males and females were used. Animals were shipped at 20-25 days depending on the specific experiment. Animals were received in filter-top containers and upon arrival, were placed in individual polycarbonate cages in an isolation room. Researchers and animal care personnel donned caps, gowns, and latex gloves when in the animal quarters. The rooms were temperature and light controlled. Animals were allowed free access to food (standard rat chow) and water. The conditions in the animal quarters were controlled to maintain virus antibody free conditions, and the vivarium draws blood from sentinel animals every six months to ensure that known pathogens have not been introduced.

All experimental animals were permitted to acclimate to the facility for approximately one week before any experiments were begun. Each animal was weighed approximately every other day (Mondays, Wednesdays, and Fridays). Failure to gain weight or loss of weight was considered to signal the probable onset of diabetes. Diabetes was verified by glucosuria detected with a paper test strip and by a blood glucose level above 200 mg/dl. Animals diagnosed as diabetic were euthanized by nembutal overdose followed by exsanguination via cardiac puncture.

Serum glucose, estimated for purposes of diagnosing diabetes, was done by first prepping the tail of the animal with alcohol and snipping the tip with sterile scissors. The drop of blood at the tip of the tail was applied to a test card and read in a glucose meter (LifeScan®, Johnson and Johnson, New Brunswick, NJ). Some animals were terminated prior to becoming overtly diabetic and these animals were anesthetized and exsanguinated by cardiac puncture. Serum was separated from the clot and glucose was measured by the glucose oxidase–glucose peroxidase method [10] using a commercial kit (Sigma Chemical Company, St. Louis, MO) in accordance with the manufacturer's directions.
Evaluation of splenic follicles was done on hematoxylin-eosin stained sections. Spleens from different animals were observed at the same magnification on a microscope equipped with a video camera. The diameter of the cortical and follicular regions of each follicle was measured directly from the video monitor. Vernier calipers were used for these measurements and each follicle (cortex and medulla) was measured in two directions at right angles.

Continuous data were analyzed by Student's t-test and the results of insulin secretion were evaluated by ANOVA (general linear model for completely randomized 2-factor conditions) followed by re-evaluation using Tukey's HSD (honestly significant difference) [11] performed using PC-SAS. Life table analysis was performed as described by Ingelfinger [12]. Significance was defined in all cases as p < 0.05.

Results

Because gliotoxin is known to interfere with functional aspects of macrophages [13,14] and polymorphonuclear neutrophilic leukocytes [14,15], we wished to determine if the compound may have an adverse effect on pancreatic cells which would exacerbate rather than ameliorate diabetes. We exposed RIN 38 insulinoma cells to various concentrations of gliotoxin and determined cell viability and insulin secretion, comparing these findings to those from gliotoxin-free controls. Two separate (gliotoxin-free) controls were used. One was a methanol-containing control which consisted of an amount of methanol equal to that present in the gliotoxin sample, and the second control contained neither gliotoxin nor methanol, but consisted only of a volume of culture medium equal to the volume of gliotoxin added to the test wells.

Gliotoxin had little effect on viability as determined by trypan blue dye uptake at concentrations of 0.5 or 1 µg/ml. However, 5 µg/ml gliotoxin appeared to be lethal for RIN 38 cells. Fig. 1 shows the release of insulin from this same study. In cultures exposed to 5 µg/ml gliotoxin, insulin release was not physiologic, but due to cytology, based on the trypan blue results. Also seen in Fig. 1 is apparent systematic variation in insulin secretion relative to gliotoxin concentrations of 0.5 and 1.0 µg/ml. Therefore, ANOVA for the low glucose condition was conducted and was non-significant (p = 0.069). However, the followup Tukey HSD evaluation showed that gliotoxin-treated RIN 38 cells produced about 30% less insulin than their respective controls. Statistical analysis of cells in the high glucose incubation medium provided virtually identical results.

We determined from this experiment that test animals should not receive more than 1 µg of gliotoxin per g of body wt at any one time, despite the fact that
Fig. 2. Peripheral blood leukocyte counts from non-diabetic rats treated with gliotoxin compared to vehicle (glycerol) treated controls. Diabetes-prone BB rats (four males and four females) were treated with ip injections of gliotoxin (1 μg/g body wt 3 times weekly for a total of 10 doses) and an equal number of animals were treated with vehicle (sterile glycerol). Blood from the tail vein was collected and diluted in white blood cell counting fluid (Unopette, Becton Dickenson, Rutherford, NJ) and counted in a hemocytometer. Statistical comparison employed Student's t-test.

this cell culture experiment would not enable us to predict systemic toxicity. We therefore selected a dosage schedule of three ip injections per week of gliotoxin (1 μg/g body wt).

Previous studies from our laboratory suggested that peripheral blood leukocytes were particularly sensitive to exposure to gliotoxin [15]. Therefore, we treated rats of both sexes for 10 days with gliotoxin and compared the peripheral blood white cell counts in gliotoxin-treated animals to those in control animals that had been treated with ip injections of glycerol three times per week. As shown in Fig. 2, the animals treated with gliotoxin did not become neutropenic after 10 doses.

In addition, we followed the weight gain of rats treated with gliotoxin as a further indicator of systemic toxicity; the results are summarized in Fig. 3. Weight gain was followed only until 65 days of age, because after 65 days, BB rats begin to become diabetic, which will result in precipitous weight loss. Weight gain was not significantly affected among treated animals, which provided a further indication that gliotoxin was not overtly harmful to the animals. Observation of treated animals also failed to reveal any subjective changes in activity or appearance of the fur, conjunctiva, or feces.

To determine if gliotoxin could alter the development of diabetes among diabetes-prone animals, we plotted the incidence of diabetes among gliotoxin-treated and vehicle-treated rats. Injections were begun at 30 days of age and continued until diabetes developed, or to day 120 for those animals that did not become diabetic. The results of this evaluation are provided in Fig. 4, which demonstrates that gliotoxin treatment delays the development of diabetes in this animal model. Life table analysis applied to this experiment revealed that the effect of gliotoxin was significant (p < 0.05).

In a separate study, we measured serum glucose levels in diabetes-prone rats treated with gliotoxin (from day 30 to day 90) to those of a similar group of animals treated with vehicle. These animals were euthanized prior to the development of clinical diabetes, and along with serum, spleens were collected for histologic evaluation. As shown by Fig. 5, animals treated with gliotoxin maintained lower blood glucose levels compared to controls as long as the animals were free of clinical diabetes.

Since gliotoxin administered according to the dosage schedule we selected failed to completely prevent clinical disease, we wished to determine if earlier treatment could further reduce the rate of diabetes in experimental rats. When ip gliotoxin treatments were begun at 20 days of age instead of 30 days, the beneficial effect was lost and the incidence of diabetes among experimental and control animals was identical (data not shown). Thus, while gliotoxin apparently could prevent diabetes, it needed to be introduced within a certain window of vulnerability.

Because diabetes is known to be an immunologic process in BB rats [16], we obtained spleens from the pre-diabetic animals treated with gliotoxin (from day 30 to day 90) as well as from control animals treated with vehicle. We examined the splenic follicles in hematoxylin-eosin stained sections and measured the diameters of the cortical and medullary regions. As shown in Fig. 6, gliotoxin treatment resulted in a significant decrease in the ratio of the cortical area to
the medullary area. In general, the lymphocyte-rich white pulp follicles seemed to be decreased in prominence among the gliotoxin-treated animals, with the greatest decrease being due to loss of the outer layer which is ordinarily rich in T lymphocytes.

Discussion

Various immunologic interventions have been considered as potential preventative therapies for autoimmune diseases, but the present study is the first to evaluate gliotoxin in this role. We were successful in decreasing the incidence and delaying onset of diabetes in the experimental animals, although different dosages, dosing schedules, routes of administration, or other modifications to the treatment regimen might have produced even better results. The initial positive results obtained in this study suggest that the cost and time required to conduct studies of dosage, routes of administration, and even testing of congeners may be
**Fig. 4.** The effect of gliotoxin treatment on the development of diabetes in diabetes-prone BB rats compared to vehicle-treated controls. Two groups of 10 rats (both sexes) were administered gliotoxin (1 µg/g body wt, ip, 3x/week beginning at 30 days of age and continuing to the end of the study) or the vehicle (sterile glycerol). Animals that became diabetic were euthanized as soon as diabetes was confirmed. Comparison employed life table analysis which showed that gliotoxin treatment delayed or prevented development of diabetes.

**Fig. 5.** Blood glucose levels among gliotoxin-treated compared to vehicle-treated rats. Diabetes-prone BB rats were treated with gliotoxin (1 µg/g body wt, ip, from 30 to 90 days of age). Control rats received ip injections of sterile glycerol. All rats were clinically non-diabetic as indicated by continued weight gain. Glucose was measured in serum by the glucose oxidase-peroxidase procedure. Error bars show SEM, and comparison was made by Student's t-test.

worthwhile in future work. We did try beginning treatments earlier, at 20 days instead of 30 days. But it was clear that earlier treatment was not effective, which is in accord with prior observations on BB rats in which it has been shown that there seems to be a time around 30 days of age when intervention is effective [17], which is sometimes referred to as the "window of vulnerability."

In addition to demonstrating that gliotoxin was able to delay or prevent the onset of disease, we showed that it also kept the glucose levels below those seen in controls even during the pre-diabetic period. This study of glucose levels adds additional support to the observations of delayed onset or decreased incidence of diabetes in treated animals. It is unlikely that the reduction of blood glucose was due to enhanced insulin release or regeneration of pancreatic beta cells, since the results with RIN 38 cells show mild compromise of the beta cells. In 1998, Tuch and co-workers [18] also demonstrated that gliotoxin was mildly toxic to human fetal pancreatic explants that were transplanted into nude mice. Thus, it is more likely that the lower blood glucose in gliotoxin-treated animals is due to interdiction of the autoimmune disease process.

Both rats and mice (NOD mice) have been used as experimental models for type I diabetes and each has advantages and disadvantages. However, neither is a perfect model of diabetes in humans. Consequently, the fact that the gliotoxin treatment proved beneficial in rats does not imply that it would be of value in humans. Moreover, the autoimmune processes in diabetes-prone BB/Wor rats and NOD mice are not identical. The rats used in this model are intrinsically lymphopenic and are subject to autoimmune problems (thyroiditis) and are susceptible to infections as well [16]. Despite the intrinsic immunologic defects in BB/Wor rats, this study implies that further immunologic changes are induced by gliotoxin. While not including an investigation of specific lymphocyte subsets in this study, we did note that splenic white pulp of the follicular cortex seemed to be decreased in volume by gliotoxin treatment. Morphometric observation of splenic sections is inexact.
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Fig. 6. Spleen morphometric evaluation in gliotoxin-treated and control animals. Gliotoxin-treated and control animals surviving until 90 days of age without becoming diabetic were euthanized and spleens were removed and 10 μm sections cut and stained with hematoxylin and eosin. Follicles from at least eight low power fields per spleen were visualized using a video camera-equipped microscope and projected on a video monitor. The thickness of medullary and cortical regions was measured directly on the video monitor. Follicles were evaluated if the section appeared to pass near the center of the follicle. Data are represented as the thickness of the cortex divided by the radius of the medulla. The average and SEM are shown for each of the treatment conditions. The numbers of follicles evaluated are noted on the figure.

and does not indicate the follicular volume because sections may not always pass through the center of the follicle. But we reasoned that any sections which did not precisely cross the center of the follicle would only serve to make the cortex appear larger. Thus, any error intrinsic in the method would tend to overestimate the size of the cortex, so that if anything, the decrease in cortical volume associated with gliotoxin treatment may actually be underestimated by our technique. We found the splenic cortex to be decreased, indicating that the predominant effect of gliotoxin may be on T lymphocyte–rich areas of the spleen, suggesting an appropriate direction for future work.

One of the important future directions for this work will be to address the mechanism of gliotoxin action in BB rats. Gliotoxin is known to be an inducer of apoptosis in lymphoid cells [19–21], and apoptosis of specific clones is involved in preventing autoreactive clones from surviving. McMinn and co-workers found that gliotoxin may also increase suppressor cell activity in mice [22]. Future studies will need to determine which cell types in BB rats are affected by gliotoxin in order to clarify its mechanism of action in this context.

Any immunotherapeutic regimen needs to balance the beneficial effect on the immune system against other types of toxicity. Limited toxicologic information is available on gliotoxin, but the results reported here suggest that its overt toxicity to animals is minimal. Although weight gain in the experimental animals treated with gliotoxin and the overall health of the animals did not appear to be compromised, the gliotoxin did have some negative effects on cell culture. While cell culture studies are not sufficient to predict the benignity of the compound, they do provide information to guide our dosing schedule. Ultimately, it will be necessary to consider the effects of gliotoxin on human cells. Tuch et al [18] investigated the use of gliotoxin to make grafts less immunologically reactive and noted some toxicity. At present, it is unknown whether human cells are more or less susceptible than rat cells to the potential toxic effects of gliotoxin, and this will need to be evaluated in future studies before the possibility of gliotoxin as a new drug entity may be considered.

Although we began this study with the thought that gliotoxin or a related compound might be useful as an alternative to cyclosporin A, it seems likely that its immediate usefulness will be as an immunologic reagent that could help provide a better understanding of autoimmune diabetes and perhaps other autoimmune phenomena. Future studies will evaluate specific lymphocyte populations affected by the gliotoxin treatment in order to identify where in the development of diabetes gliotoxin is having its effect. Global inhibitors of immunity are less valuable than specific inhibitors of some limited feature of lymphocyte function or inhibition of certain lymphocyte subsets. The data obtained thus far indicate that gliotoxin is able to diminish the incidence and extend the time of disease onset in BB/Wor rats without apparent compromise to the overall health of the experimental animals. Even if these observations cannot be translated into future therapies, gliotoxin
may be viewed as a useful immunologic reagent that could add new details to our understanding of autoimmunity, particularly if the effect of gliotoxin on the immune system proves to be specific and not global.

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References