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Immunologic Effects of Gliotoxin in Rats: Mechanisms for Prevention of Autoimmune Diabetes Mellitus

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Abstract. Various fungal products, such as gliotoxin (GT), have immunomodulating activity, a fact exploited previously by our group for prevention of autoimmune diabetes mellitus in BB/Wor rats. To understand better the immunologic effects in GT-treated rats, splenocytes from 65-day-old prediabetic diabetes-prone rats were phenotypically characterized after chronic treatment with GT. A parallel study examined the direct effects of GT on splenocyte preparations incubated with the mycotoxin. In vitro treatment of splenocytes with GT revealed relative decreases in $CD4^+$ and increases in $CD8^+$ T-cell subsets, whereas in vivo treatment with GT did not result in detectable alterations in relative $CD4^+$ and $CD8^+$ cell subsets. We were unable to show significant effects on NK cells or MHC class II cells. However, in vitro and in vivo GT treatments significantly enhanced the detectable RT6 surface marker, a key regulatory element in autoimmune diabetes pathogenesis. This study showed that GT selectively affects certain lymphocyte subsets, possibly through the mechanism of apoptosis, which was increased in vivo as well as in vitro. *(received 22 May 2000, accepted 26 July 2000)*

Keywords: diabetes mellitus, mycotoxin, autoimmunity, T lymphocytes, apoptosis

Introduction

In a previous study, we reported that chronic administration of gliotoxin (GT), an epipolythiodioxopiperazine mycotoxin, to diabetes-prone (DP) BB/Wor rats resulted in a decreased incidence and delayed onset of diabetes [1]. Because GT is an immunosuppressive or immunomodulating substance [2,3] and because diabetes in this rat model is an autoimmune phenomenon [4], we designed the present study to evaluate the effects of GT on the immune status of rats.

Numerous immunosuppressive drugs are known to prevent diabetes in animal models, and cyclosporin A has shown activity in preventing diabetes in clinical studies in humans [5]. However, immunosuppressive compounds may possess broad toxicity toward the immune system and carry the risk of serious side effects. Therefore, drugs that have specific and limited targets within the immune system may provide more clinical utility than drugs that lack specificity. One purpose of this investigation was to discover whether GT had global or limited effects on the immune systems of rats. Another purpose was to gain information about the mechanisms of GT's immunomodulating effects.

The general approach of this investigation was to examine lymphocyte subsets after treatment with GT in vivo and in vitro and determine if the effects were altered when cultured lymphocytes were mitogenically activated. Because regulation of the immune system often involves apoptotic deletion of potentially harmful clones, we also examined the role of GT in generating apoptotic cells in rats.

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Materials and Methods

Experimental animals. Equal numbers of male and female DP rats were purchased from the National Institutes of Health's central breeding colony at the University of Massachusetts Medical School (Worcester, MA). DP rats were shipped in filter crates and were maintained in isolation from other animals and humans (viral antibody–free conditions). Our vivarium performs periodic serologic testing on sentinel animals to ensure that specific pathogens have not entered the facility. Upon arrival, animals were caged individually and given one week to become acclimated to the animal facility. The environment was temperature- and light-controlled (12 hr light/12 hr dark). Animals were provided food and water ad libitum. Water bottles were sterilized before reuse, but food, water, and bedding were not. Standards for care of these rats have been published by Olsen et al [6]. Investigators and animal caretakers donned masks, caps, gloves, and gowns before entering the animal rooms to avoid contamination.

In vitro study. DP rats that did not receive GT (pre-diabetic, untreated rats) were killed at 65 days of age, and splenic cells were isolated as described below. Half of the splenic cells were incubated for one hr with 1 µg/ml GT (a dose that was selected on the basis of cell-culture data in our prior report [1]) at 37°C in a CO_2 (5%) incubator, and the other half were cultured in GT-free cell-culture medium as controls. The suspending vehicle, ethanol, was added to each control sample in an amount equal to that present in GT-treated samples. Both GT-treated samples and controls were divided into two aliquots. One aliquot received 10 µg/ml concanavalin A (ConA) and another remained unstimulated, yielding four different preparations of splenic cells: GT-treated cells; ConA-stimulated GT-treated cells; controls (no GT and no ConA); and ConA-stimulated controls. These cells were fluorescent-labeled with monoclonal anti-lymphocyte surface marker antibody, analyzed by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-labeling) method for lymphocyte apoptosis, or used in proliferation tests, each described below.

In vivo study. For this study of lymphocytes from GT-treated rats, experimental animals were placed into two groups. Half of the DP rats were chronically treated with GT from 30 days of age until 65 days of age. The control animals received glycerin (vehicle) injections. GT-treated animals received 1 µg/g body weight three times weekly by intraperitoneal injection; the volumes injected ranged from 0.1 to 0.25 ml, depending upon the weight of the animal. All rats were monitored for weight gain to verify that they were not becoming diabetic, and were killed at 65 days of age. Spleens and pancreases were recovered and used as described below.

Splenocytes from GT-treated rats and control rats were divided into two aliquots. One aliquot was stimulated with 10 µg/ml ConA, and the rest of the cells remained unstimulated. Four different samples were prepared: splenocytes from GT-treated rats; splenocytes from GT-treated rats with ConA stimulation; splenocytes from control rats; and splenocytes from control rats with ConA stimulation. Samples were used for surface marker labeling, apoptosis analysis, and proliferation testing as described below.

The concentration of ConA used in this study was based on a titration of effect on spleen cell metabolic activity demonstrated by Alimar Blue (Sensititre/Alimar, Sacramento, CA) reduction. We found that 10 µg/ml ConA was more effective than either 1 or 5 µg/ml with cells from DP rats (data not shown).

Isolation of splenic leukocytes. At 65 days, experimental animals were euthanized by CO_2 inhalation. A midline incision was made to expose the spleen, which was aseptically removed and placed in 10 ml of Dulbecco's phosphate buffered saline (PBS) on ice for transport from the vivarium to the laboratory. In the laboratory, splenic tissue was minced and further disrupted by passage through a sterile 80 mesh tissue sieve in a laminar-flow hood. Cells were further dispersed by repeated aspiration and expression through a sterile 21-gauge needle, washed twice with 15 ml of Dulbecco's PBS, and recovered by centrifugation at 1000 rpm for 4 min. Isolated leukocytes were suspended in Eagle's MEM with 10% FBS and, unless otherwise noted, incubated in a 25 ml tissue culture

flask in CO_2 (5%) at 37°C to remove adherent cells. Viable cell counts of non-adherent cells were determined by 0.4% trypan blue staining. Hemocytometer counts of trypan-stained cells showed >95% viability.

Lymphocyte surface marker labeling. Splenic lymphocyte subsets were stained with antibodies listed in Table 1, which also identifies the isotype controls used. Prior to analysis of lymphocyte surface markers, each antibody was titrated to determine an appropriate antibody dilution. Splenocytes from normal rats were used for titration. Anti-CD4 and anti-CD8 mAbs were diluted 100-fold in PBS, and other mAbs were diluted 50-fold. Splenic lymphocytes were harvested by centrifugation and resuspended to a concentration of 10^6 cells/ml in Dulbecco's PBS with 1% FBS. Splenic lymphocytes were added to a series of tubes (10^5 cells/tube) and incubated with equal volumes of FITC- and PE-labeled mAbs at appropriate dilutions for 30 min on ice. Stained cells were washed three times with assay buffer (1 mg/ml sodium azide in PBS). The secondary antibody was applied to unlabeled anti-RT6 mAb after primary antibody binding. The labeled cells were fixed by adding 500 µl of 4% paraformaldehyde. The fixed cells were stored at 4°C overnight for flow cytometry evaluation.

Table 1. Specifications and sources of the antibodies in the panel used to immunophenotype splenic cells.

Marker (Clone)	Species	Source*	Label
CD4 (OX35)	Mouse anti-rat	Pharmingen	PE**
CD8 (OX8)	Mouse anti-rat	Pharmingen	FITC**
NK (3.2.3)	Mouse anti-rat	Harlan	FITC
RT6 (P4/6)	Mouse anti-rat	Bisource	Unlabeled
PanT (OX19)	Mouse anti-rat	Pharmingen	PE
MHCII (OX17)	Mouse anti-rat	Biosource	FITC
Secondary	Goat anti-mouse	Sigma	FITC
Isotype control	Mouse IgG1	Sigma	FITC
Isotype control	Mouse IgG2	Sigma	PE

* Pharmingen, San Diego, CA; Harlan Bioproducts, Indianapolis, IN; Biosource, Camarillo, CA; Sigma, St. Louis, MO.

** PE, Phycoerythrin; FITC, Fluorescein isothiocyanate.

Flow cytometry. Data collection employed the FACScan apparatus (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Cell Quest software was used to record, convert, and analyze data. For each sample, the flow cytometer scanned at least 10,000 cells and recorded four parameters: forward light scatter (FSC), side light scatter (SSC), FITC staining (FL1), and PE staining (FL2). The lymphocyte populations were electronically selected from FSC and SSC characteristics and replotted into a dot plot or a histogram plot, which included the percentage of FITC- or PE-labeled cells among the lymphocyte populations. For analysis of $RT6^+$ cell data, the intensities of fluorescent staining for each cell were recorded as channel numbers, and the mean channel number representing the average fluorescent intensity of all cells with any RT6 surface stain was calculated. The data were normalized by dividing mean channel numbers for experimental preparations by the average channel number for all samples analyzed during a given flow cytometry session, to yield a mean channel index.

Leukocytic metabolic activity. Splenic lymphocytes, obtained as for other experiments, were suspended in MEM (Sigma Chemical, St. Louis, MO) with 10% fetal bovine serum. Leukocytic suspensions with ConA (10 µg/ml) were added to a 96-well microtiter plate (10^5 cells/well). Each test was performed in triplicate. Cells in control wells were incubated with MEM only. All wells had 50 µl of the indicator Alimar Blue. Incubation was at 37°C in 5% CO_2 for 72 hr. Proliferation data (reflected by metabolic dye reduction) was

determined from absorbance readings at 590 nm; a stimulation index was calculated (A_{590} with mitogen/ A_{590} without mitogen).

Apoptosis. Splenic lymphocytes isolated from GT-treated DP rats or controls, splenic lymphocytes treated with GT in vitro, and control lymphocytes were stained by the TUNEL method with Oncor Fluorescent Apop-Tag Kit (Oncor, Inc., Gaithersburg, MD) and analyzed by flow cytometry to determine the proportion of lymphocytes undergoing apoptosis. Lymphocytes were obtained from experimental animals and fixed in ice-cold 1% paraformaldehyde in PBS at pH 7.4 for 15 min, followed by three washes in PBS. Fixed leukocytes were resuspended in 70% ice-cold ethanol and kept at -20°C up to 5 days. Enzyme reactions and staining followed the manufacturers' directions for staining fragmented DNA. Positive and negative controls were prepared and analyzed by flow cytometry to establish the fluorescent intensities (FL1 and FL2) associated with apoptotic and non-apoptotic cells.

Statistical analysis. Data were evaluated by t-test (Sigma Plot, Jandel Corp., San Rafael, CA). The in vitro studies used aliquots of identical cell preparations, allowing statistical analysis by means of paired t-test. The in vivo studies employed the unpaired t-test.

Results

Cells with MHC class II surface proteins. The effect of GT on MHC II–positive leukocytes (potentially antigen-presenting cells) was investigated following in vitro GT treatment of splenocyte suspensions. These cell suspensions were not pre-incubated in plastic flasks to remove adherent cells, as in subsequent studies of lymphocytes. Initially, flow cytometry data for MHC II–stained cells was evaluated by gating on a region containing lymphocytes, which failed to show a significant effect of GT on this populations of cells. However, when we chose a larger and more granular population than those contained in the lymphocyte gate, GT did have a significant effect on MHC II–positive cells (Fig. 1). ConA widened the difference between GT-treated and control cells. However, the number of cells included in this larger gate was relatively small, so it is difficult to consider this finding conclusive with regard to antigen presentation.

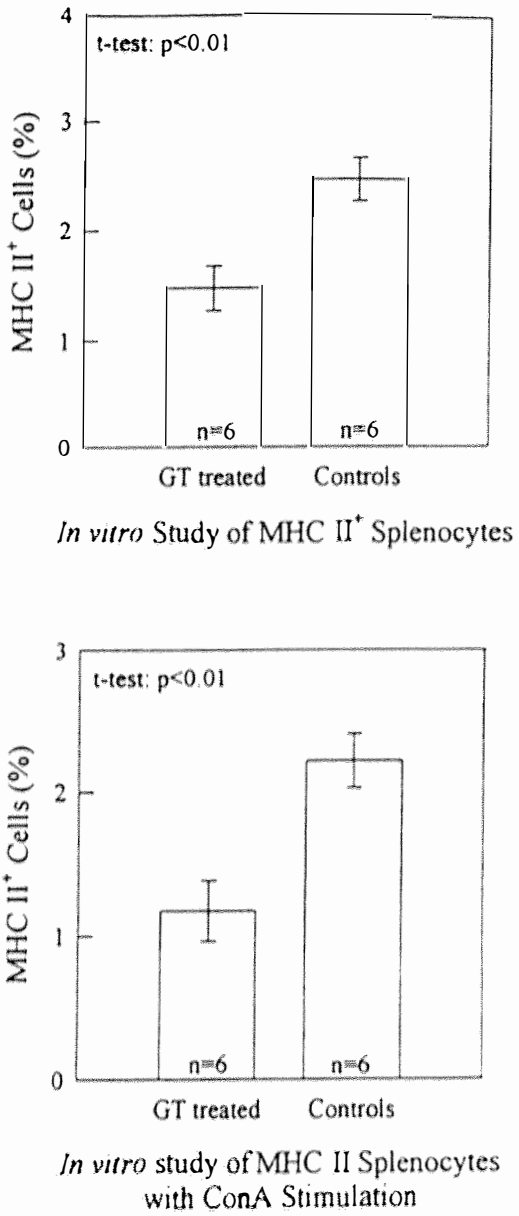


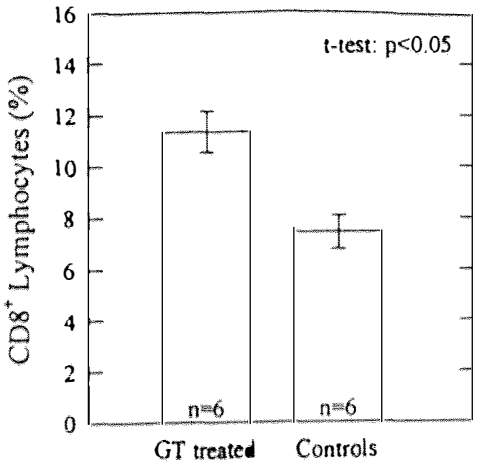
Fig. 1. Percent of cells staining positively for MHC II are indicated on the y axis. Error bars are SD in all figures. One hr of GT treatment (1 µg/ml) of rat splenocytes from prediabetic rats reduced the percentage of cells bearing the MHC class II marker significantly (upper graph), and in the presence of ConA this effect persisted (lower graph).

NK cells. NK cells have been implicated in damage to pancreatic islet β cells. Isolated splenocytes from GT-untreated, 65-day-old, nondiabetic DP rats (with or without ConA stimulation) were incubated for 1 hr with GT and labeled with FITC-conjugated anti-NK mAb. GT treatment increased the relative NK cell percentage, but no difference was seen with ConA stimulation (data not shown), suggesting that GT does not exert its effects on IDDM through NK suppression. NK staining of lymphocytes from rats treated with GT compared to the untreated controls showed no difference, regardless of whether or not the cell suspension was exposed to ConA.

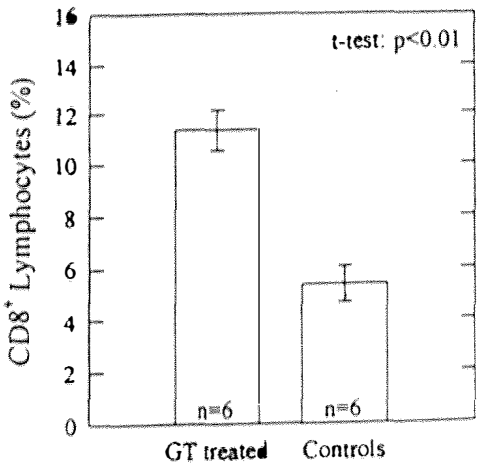
CD5⁺ lymphocytes. Because prior observations revealed GT-mediated morphological changes in splenic white pulp pointing to an effect on T cells [1], CD5 stain was used to determine the percentage of T cells in lymphocyte preparations from both in vitro and in vivo experiments. Lymphocytes from non-GT-treated rats were exposed to GT or vehicle and aliquots were stimulated with ConA or left unstimulated. This PanT staining did not show a response to GT treatment in vitro, and ConA had no effect on these data. GT treatment of prediabetic rats showed no difference between CD5 staining of control and GT-treated animals. ConA stimulation had no additional effect on these data.

CD8⁺ lymphocytes. While PanT markers showed no sensitivity to GT, subsets of lymphocytes did. $CD8^+$ T splenic lymphocytes were evaluated in a method similar to that described for $CD5^+$ cells. Lymphocytes incubated with GT in vitro without ConA stimulation showed a relative increase in $CD8^+$ lymphocytes (Fig. 2), and this difference was enhanced by ConA stimulation to 53% above counts obtained in the absence of GT treatment. The results obtained from in vivo treatment with GT were not as pronounced, and ConA stimulation did not create a distinction between in vivo GT-treated $CD8^+$ lymphocytes and controls (data not shown).

CD4⁺ lymphocytes. $CD4^+$ T lymphocyte subsets were examine in an in vitro study that involved 1 hr incubation of splenic lymphocyte suspensions with GT with or without ConA stimulation. As shown in



In vitro Study of $CD8^+$ Splenic Lymphocytes



In vitro Study of $CD8^+$ Splenic Lymphocytes with ConA Stimulation

Fig. 2. Lymphocytes suspensions treated for 1 hr with 1 μg/ml GT showed a relatively larger proportion of cells staining with the anti-CD8 antibody (upper panel) and this effect was accentuated by introducing ConA (lower panel).

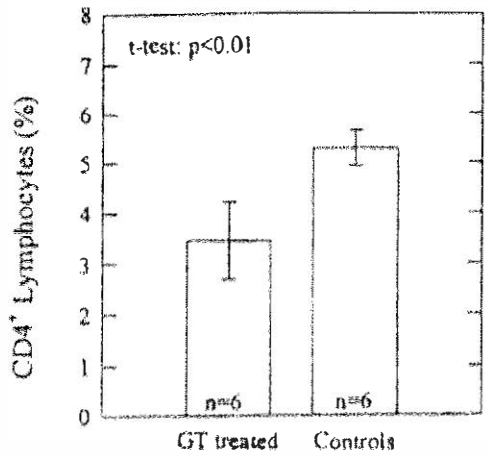
Fig. 3, GT-treated lymphocytes contained fewer $CD4^+$ cells than did populations of untreated control cells. ConA enhanced the difference between GT-treated lymphocytes and GT-untreated controls. Control cells not treated with GT had more than twice the percentage of $CD4^+$ T lymphocytes that GT treated cell suspensions had. When GT was administered chronically to DP rats, no clear effect of GT on splenic $CD4^+$ populations was found, and ConA did not affect this relationship (data not shown).

RT6⁺ lymphocytes. Evaluation of $RT6^+$ cells was conducted according to both in vitro and in vivo protocols as with other spleen cell phenotypic markers; however, fluorescent intensity, rather than cell number, measured RT6 cell staining. This technique was required by the lack of a distinct RT6 cell population that could be separated from non-staining lymphocytes. The amount of RT6 staining was significantly increased among cell suspensions treated with GT (Fig. 4), and while ConA did not enhance this difference, the difference remained significant for ConA-treated cell suspensions.

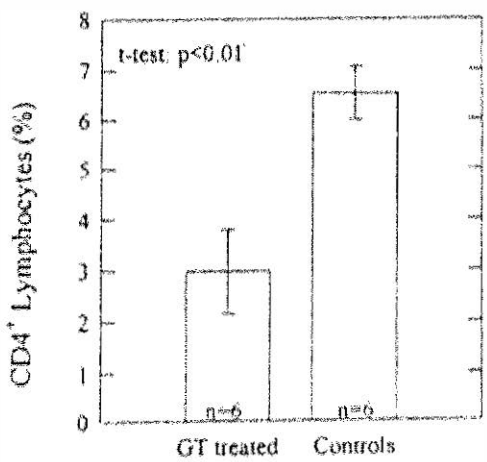
For the study of RT6 in vivo, rats received GT injections or control injections. Analyses were done as in the in vitro study. Fig. 4 also shows that the mean RT6 staining index was 1.156 in GT-treated samples and 0.856 in GT untreated controls, a difference which was statistically significant. ConA treatment did not eliminate this significant effect.

Metabolic activity. GT may have an effect on immune cells by inhibiting metabolic or proliferative activity of lymphocytes. The metabolic activity of leukocytic suspensions was measured with the Alimar Blue technique and, as shown in Fig. 5, a significant reduction in cellular metabolic activity resulted from exposure to GT. This effect was not reversed by the addition of ConA.

Apoptosis analysis. The level of apoptosis was studied both in cells derived from GT-treated animals and from untreated animals whose spleen cells were treated with GT in vitro. The apoptosis of DP rat lymphocytes was also studied with or without ConA stimulation, since apoptosis may be enhanced or suppressed when superimposed on cytokine production, receptor

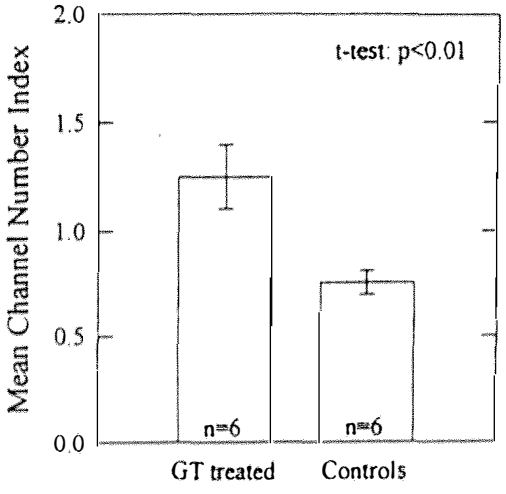


In vitro Study of $CD4^+$ Splenic Lymphocytes

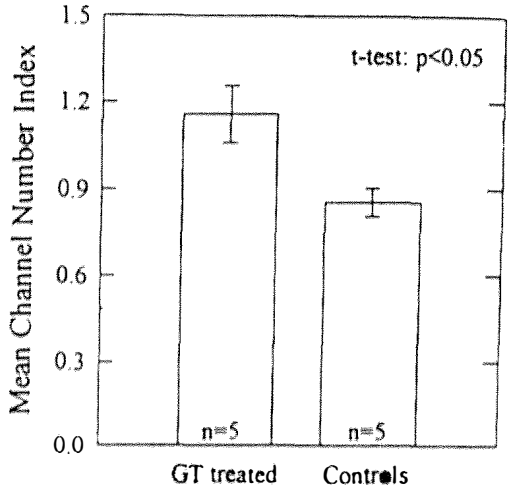


In vitro Study of $CD4^+$ Splenic Lymphocytes with ConA Stimulation

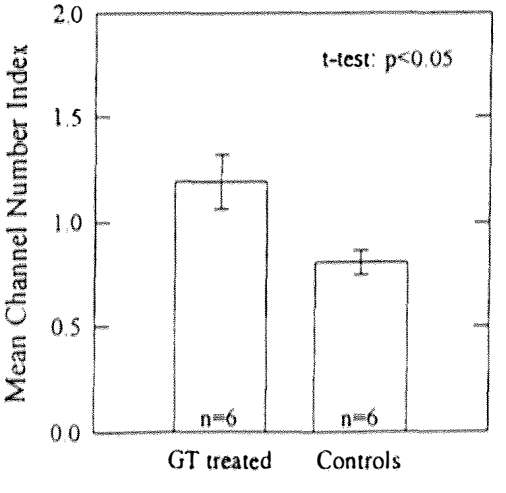
Fig. 3. Lymphocytes obtained from non-GT-treated rats were exposed to GT (1 μg/ml for 1 hr) in vitro. This resulted in a significant decrease in CD4-staining cells compared to untreated controls (upper panel). ConA accentuated this difference (lower panel).



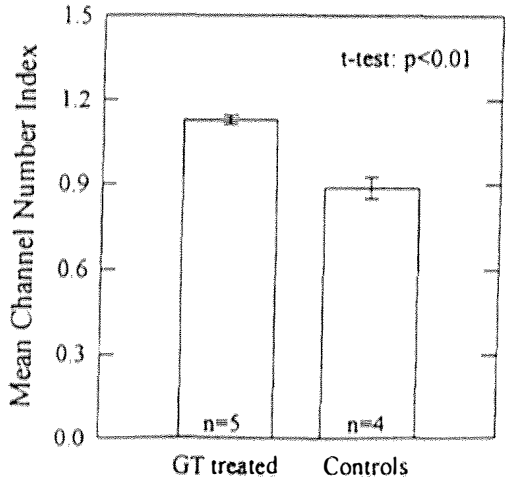
RT6$^+$ T Cell Staining of *in vitro* Study



RT6$^+$ T Cell Staining of *in vivo* Study

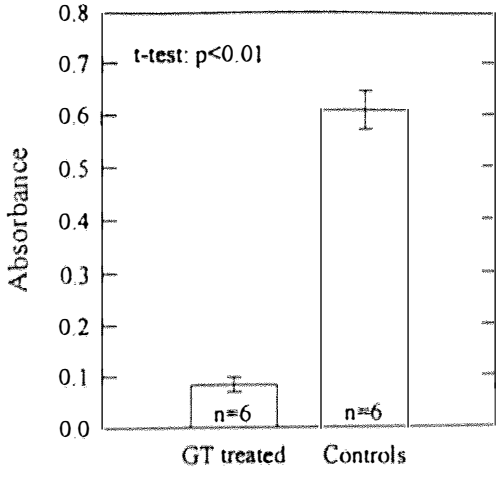


RT6$^+$ T Cell Staining of *in vitro* Study with ConA Stimulation

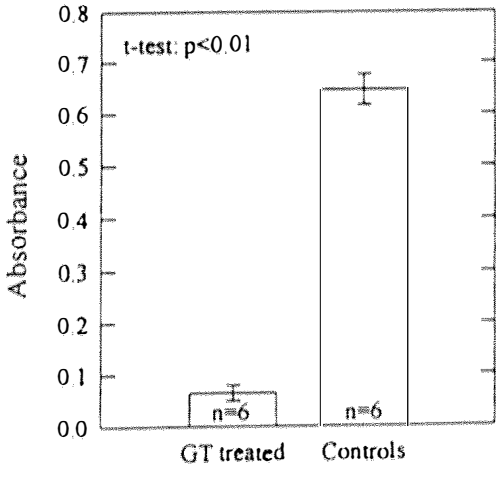


RT6$^+$ T Cell Staining of *in vivo* Study with ConA Stimulation

Fig. 4. RT6$^+$ cells exist in very low abundance in diabetes-prone rats. Therefore, RT6 marker was measured by means channel index (see methods) only for those cells staining for RT6. As shown in the upper left panel, GT treatment of diabetes prone rats (in vivo) increased RT6$^+$ staining and ConA did not alter this relationship (lower left panel). For the parallel in vitro experiment, the difference between GT-treated and control cells was also significant, and the difference was not abrogated by ConA stimulation.



In vitro Metabolism Study of Splenic Leukocytes



In vitro Metabolism Study of Splenic Leukocytes with ConA Stimulation

Fig. 5. Metabolic activity of leukocyte cultures was based on Alimar Blue color change. Increased absorbance at 650 nm (y axis) indicates increased metabolic activity. GT treatment of rat leukocytes significantly decreased oxidative metabolism (upper panel) and ConA failed to restore GT-treated cell metabolism (lower panel).

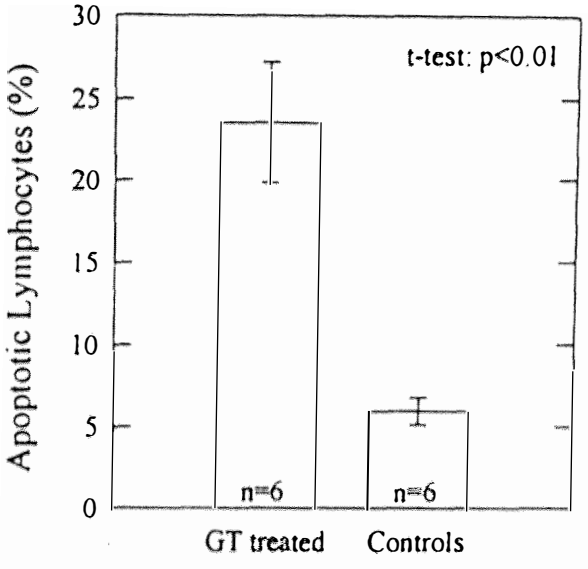
expression, and T-cell proliferation. As illustrated in Fig. 6, GT-treated splenic lymphocytes showed 23.6% apoptosis, a level significantly higher than the 6.0% seen in untreated controls. When repeated with ConA stimulation, this same experiment showed a 4-fold difference, as it did without ConA ($p < 0.01$).

In vivo GT treatment pr vided results which were not as pronounced as with in vitro GT treatment. Splenic lymphocytes from GT-treated DP rats showed 15.67% apoptosis, compared to 7.50% in untreated controls (Fig. 6). ConA stimulation of lymphocytes recovered from GT-treated or control rats showed a significant difference in apoptosis levels, with 6.3% apoptosis in splenic lymphocytes from GT-treated rats and 2.6% apoptosis in those from untreated controls.

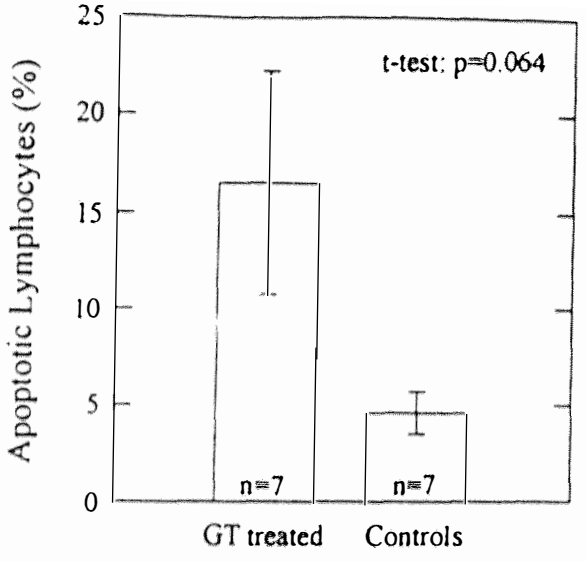
Discussion

Although various methods to lessen the immunological effects in autoimmune diabetes have been studied, none has proven ideal for prevention or treatment. Studies have indicated that GT has inhibitory effects on the immune system [7,8,9] and decreases diabetes incidence in DP rats, although the mechanism has not been reported [1]. We proposed that GT may function as a selective immunosuppressive, since it appears to influence several regulatory T-cell subsets without causing the same reaction among all lymphocyte subpopulations. Such a non-global effect is important because the clinical promise of immune modulator therapy requires selective rather than universal immunosuppression.

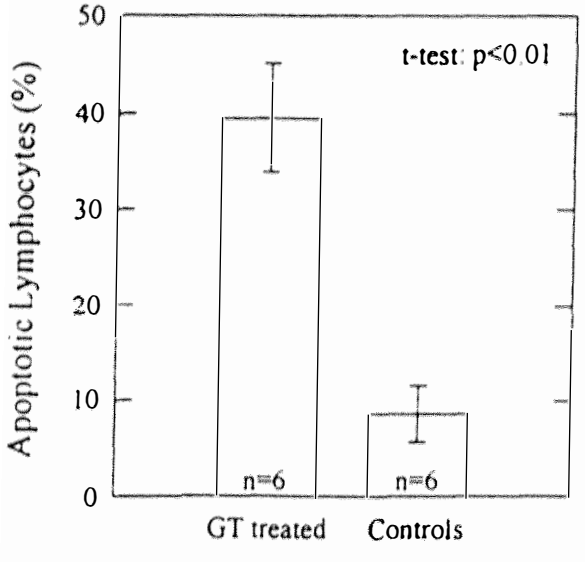
To understand GT's effects on the immune systems of DP rats, we examined lymphocyte subsets in prediabetic DP rats. These analyses were conducted on isolated cells treated with GT and on intact animals. Although isolated cells are more sensitive to GT's effects, in vivo studies are essential to establish the value of GT, as was previously shown in demonstrating its efficacy in diabetes prevention [1]. Information regarding the pharmacokinetics and pharmacodynamics of intraperitoneally administered GT was beyond the scope of this study. In vitro, we were able to control GT at a 1 µg/ml level, but in vivo we gave doses on alternate days that would not be expected to exceed 1 µg/ml. Even if a steady state were reached, we would expect that steady state level to be below the



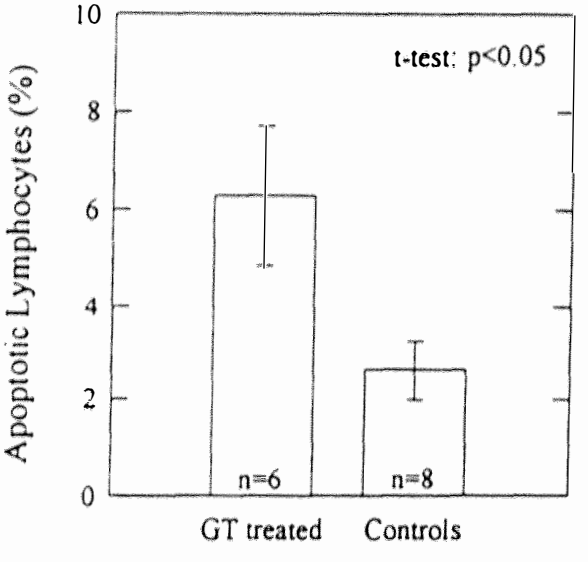
In vitro Apoptosis Study of Splenic Lymphocytes



In vivo Apoptosis Study of Splenic Lymphocytes



In vitro Apoptosis Study of
Splenic Lymphocytes with ConA Stimulation



In vivo Apoptosis Study of
Splenic Lymphocytes with ConA Stimulation

Fig. 6. Left panels depict the role of GT in inducing apoptosis in cells obtained from non-GT-treated rats. Right panels show apoptosis in GT-treated compared to non-treated rats. Percent of cells showing TUNEL staining are plotted on the y-axis. Upper panels represent experiments done without ConA and lower panels had ConA added. Apoptotic cells from GT-treated rats were not significantly increased (upper right panel) over controls, but with the addition of ConA (lower right panel) the difference became significant.

1 μg/ml level. Therefore, the fact that we observed more profound effects of GT in vitro than in vivo is not surprising. This conservative dosage schedule could be expected to have a less profound effect on lymphocyte populations than that observed when isolated cells are placed in direct contact with GT in vitro. However, in our prior study, this dosage schedule resulted in altered diabetogenesis [1].

For this study, we viewed cells in functional categories of antigen-presenting cells (APCs), cytotoxic effector cells, and regulatory cells, since any of these mechanisms may play a role in autoimmune pathogenesis. The functions are associated with cells bearing specific markers, but results must be interpreted with caution because broad categories of lymphocytes do not completely and uniquely correlate with functions.

Because GT failed to prevent diabetes when treatment was begun late in pathogenesis in our previous study [1], the present study focused on earlier intervention during the prediabetic period. ConA treatment was superimposed in this study, since this mitogen induces T-cell proliferation and enhances cytokine production and receptor expression, which may increase sensitivity of T-cell subsets to GT. We did not consider using other leukocyte stimulants such as those that preferentially affect humoral responses, since most investigators consider cell-mediated responses most relevant to the pathogenesis of diabetes.

One of the prevailing theories of autoimmune pathogenesis implicates faulty antigen presentation in diabetes development. Antigen presentation may also stimulate regulatory T-cell activation and enhance autoimmune pathogenesis. The activated regulatory T cells may also increase the activity and/or number of APCs to further enhance the autoimmune antigen presentation and the autoimmune process. For this reason, we investigated GT's effect on MHC II cells, which include APCs that may inappropriately display self-antigens as foreign and induce autoimmunity. Discernible effects of GT on MHC II cells were limited to a subpopulation that was selected to include larger and more granular cells than lymphocytes. This strategy should have excluded most B lymphocytes, which are probably of little consequence in the antigen presentation pertaining to autoimmune diabetes. This analysis showed that at least some MHC II cells were eliminated by GT, although the result was not profound, and suggested that APCs were probably not the major target for GT.

Evaluation of NK cells, which may participate in late stages of diabetogenesis, in GT-treated lymphocytes and controls showed no significant difference. This is not surprising since a previous study showed that GT treatment beginning at 40 days of age was ineffective [1], implying that effector cells acting in the late stages of pancreatic damage are probably not the target of GT's beneficial effect.

Our present study revealed changes within different T-cell subsets treated with GT. We focused on cells bearing the $CD4^+$, $CD8^+$, and $RT6^+$ phenotypes. Because T cells in general are probably too broad a category to delineate GT's effects, we were not surprised to find no clear effect on $CD5^+$ cells. $CD4^+$, $CD8^+$, and $RT6^+$ cells possess functional (such as cytotoxic) and regulatory (such as suppressor and helper) roles in normal and abnormal immune responses. $CD4^+$ T-cell percentage was decreased and $CD8^+$ T-cell percentage was increased in in vitro experiments. Similar results were obtained in vivo, but statistical significance was not attained due to the limited number of animals. Together, such results portray GT as a selective immunotoxin or immunomodulator, a key property for a useful immunotherapeutic drug.

$CD4^+$ and $CD8^+$ lymphocytes have documented roles in models of type 1 diabetes and other autoimmune diseases. Studies showed that both $CD4^+$ and $CD8^+$ T cells were required for successful adoptive transfer of disease with diabetic spleen cells into young NOD mice [10] and irradiated adult NOD mice [11, 12], but the relative roles of the two subsets remain controversial. $CD4^+$ T cells from donor NOD mice were critical for diabetes development in T cell–depleted NOD mice [13]. Purified $CD4^+$ T cells from diabetic donor spleens transferred disease at low efficiency in immunodeficient NOD mice, although purified $CD8^+$ T cells did not [14]. Other studies indicated that the role of $CD4^+$ T cells in diabetes pathogenesis was to recruit $CD8^+$ T cells as final effectors in islet β-cell destruction [15,16]. One $CD4^+$ T-cell clone (BDC-6.9) was shown to transfer diabetes without help from host B cells, $CD4^+$ T cells or $CD8^+$ T cells [17]. A second $CD4^+$ T-cell clone (BDG-2.5)

only induced diabetes when $CD8^+$ T cells were present. Different T-cell clones require different levels of accessory cell involvement. BDC-6.9 cells arise later in the disease process and can act alone. Because BDG-2.5 cells may be representative of the earliest diabetogenic $CD4^+$ T cells that require help from $CD8^+$ T cells, they would be an appropriate cell subset to investigate in future studies.

When an individual becomes diabetic, the $CD4^+$ T-cell percentage increases significantly, and the $CD8^+$ T-cell percentage declines slightly. A human type 1 diabetes study [18] indicated that $CD4^+$ and $CD8^+$ T-cell subsets were 38% and 27%, respectively, before diabetes and 44% and 26% after diabetes developed. The ratio of CD4/CD8 T cells was also increased. Our observed decrease in $CD4^+$ T cells, increase in $CD8^+$ T cells, and decrease in the CD4/CD8 T-cell ratio are noteworthy because they are consistent with a beneficial effect in autoimmune diabetes, although the effect was limited to in vitro observation.

Interestingly, the $RT6^+$ marker on mature lymphocytes is detected only at a very low level on a small number of cells in DP rats. In diabetes-resistant (DR) animals, it is present on 40-60% of mature T lymphocytes. Explanations for the $RT6^+$ T-cell subset deficiency include premature cell death and gene regulation defects. Although GT treatment did not restore normal levels of $RT6^+$ cells in DP rats, it increased the apparent amount of RT6 surface marker in vivo and in vitro. The level of fluorescent intensity was used to identify the effect on RT6 expression.

Previous investigators have underscored the importance of the RT6 surface marker among DP rats in contrast to DR rats at different ages. Neither RT6.1 nor RT6.2 was found on DP rat T cells, but these surface markers were found on T cells of other rats [19]. A soluble form of RT6 rat lymphocyte alloantigen was detected in serum of DP and DR rats by Western blot analysis, but DP rats circulated less RT6 alloantigen than did DR rats [20]. Anti-RT6.1 antibody injection into DR rats depleted >95% of peripheral $RT6^+$ T cells, but did not reduce levels of circulating T cells or the in vitro response of spleen cells to mitogen. If this treatment was started at 30 days of age, it induced diabetes in DR rats, but if started at 60 days of age, it failed to produce these effects [19].

The immunoregulatory function of the $RT6^+$ T-cell subset has been confirmed by many studies, and the absence of RT6 surface marker in DP rats was investigated by Crisa et al [21]. They discovered an mRNA encoding RT6 protein in spleen cells of DP rats, and the nucleotide sequence of this transcript revealed an intact coding region for the RT6 alloantigen. In addition, RT6 mRNA was translated in vivo, but the amount of RT6 protein in DP rat lymphocytes was less than 10% of the amount found in DR rat lymphocytes. The intact phosphatidylinositol linkage of the molecule to the cell surface was detected in DP rat lymphocytes by immunoprecipitation. These investigators concluded that defects in RT6 gene regulation or other cellular defects led to premature cell death in these animals.

We detected higher anti-RT6 immunofluorescent staining in GT-treated splenic lymphocytes, which may have resulted from enhanced expression of RT6 protein on the surface of lymphocytes after exposure to GT. Direct investigation of RT6 protein levels would be appropriate to verify this explanation. After gene transcription, a series of enzymes and regulatory proteins are involved in translation, modification, and protein targeting, and a role for GT could be postulated at various steps. Although GT treatment failed to restore $RT6^+$ T cells to levels comparable to those seen in DR and other normal rats, more RT6 protein on the lymphocyte surface was observed, which may increase immunoregulatory function and in turn result in decreased β-cell loss.

An area for future investigation is cytokine function, which has been linked with diabetes pathogenesis, and its modulation by GT. Helper T lymphocytes (Th) (mostly $CD4^+$) and cytotoxic T lymphocytes (Tc) (mostly $CD8^+$) are characterized by particular patterns of cytokine production. These patterns distinguish Th1 from Th2 cells and Tc1 from Tc2 cells. Th1 and Tc1 T cells produce IL-2 and IFN-γ, which appear to be positively related to diabetes. Th2 and Tc2 T cells produce IL-4, IL-5, and IL-10, which inhibit diabetes [22]. Some evidence from this study may indirectly link GT function to cytokine production. For example, ConA stimulation, which elicits cytokine secretion, enhanced GT-induced apoptosis. The difference in $CD8^+$ T-cell percentage between GT-treated lymphocytes and controls was widened by ConA stimulation. GT treatment may

enhance production and function of diabetes-preventive cytokines and inhibit diabetes-promoting cytokines. This relationship may be investigated in vitro and in vivo as appropriate rat anti-cytokine probes become available.

Three possible explanations for the GT-induced changes among T cells are lymphopoiesis, selective proliferation of T-cell clones, and toxic effects on specific clones. Prior studies suggested that GT probably did not have an adverse effect on stem cells [1]. We examined the ability of lymphocytes to proliferate in the presence of ConA and found that GT significantly inhibited lymphocyte metabolism in vitro and that this effect was not overcome by ConA (data not shown). In vivo GT-treated cells failed to show a significant response to ConA, raising the question of whether GT persistently blocked the proliferative ability of these cells.

The finding that GT can induce apoptosis of lymphocytes both in vitro and in vivo may explain the decreased $CD4^+$ T-cell percentage with GT treatment and identify lymphocyte elimination as a likely explanation for the changes in T-cell subsets. Some reports indicate that GT induces apoptosis in different cell types in the immune system, including macrophages [23,24], splenic lymphocytes [25], and T blast cells [23]. GT also triggers cell surface receptors and enhances lymphocyte activation [26]. This study is the only report that shows that GT can enhance lymphocyte apoptosis both in vivo and in vitro.

Apoptosis is an important mechanism by which the immune system eliminates harmful cells and controls immune responses. Although several apoptotic mechanisms have been studied, Fas and Fas ligand interaction is among the most important for T-lymphocyte apoptosis. Normally, APCs express self-antigen as a complex with MHC. This complex interacts with T-cell receptors on autoreactive T cells. Activation of these T cells induces Fas and Fas ligand gene expression. T cell–T cell interaction causes elimination of harmful T cells through Fas ligand binding to its cognate receptor [27], thereby preventing development of autoimmune disease.

We do not know the precise mechanism whereby GT promotes apoptosis. GT may activate certain types of T lymphocytes through triggering of surface receptors. This T-cell activation could increase Fas and Fas ligand expression and enhance apoptosis. A second possibility is that GT directly binds Fas protein on the T-cell surface, thereby triggering Fas receptor as Fas ligand normally would and resulting in apoptosis of target T cells. Finally, it is possible that GT, which is relatively hydrophobic, passes through the lymphocyte cell membrane and binds specific cellular components. The disulfide group of GT may bind sulfhydryl or disulfide groups on target proteins. If these GT-binding proteins are involved in regulating the cellular processes, such GT-protein interactions could enhance apoptosis [28]. Because ConA stimulation, which enhances surface receptor production, widened the difference in apoptosis between GT-treated cells and untreated controls, a direct triggering of Fas receptors by GT seems possible. Additional investigation will be required to confirm this proposed role for GT.

Because GT induced apoptosis and altered the relative abundance of certain lymphocyte subsets, we inferred that apoptosis could account for T-cell subset changes. To verify this concept, we would need to show that apoptosis occurs in the same cell subsets that we found to be decreased by GT treatment.

We have demonstrated that changes in T-cell subsets that occur with exposure to GT may be consistent with a salutary effect in inhibiting diabetes in DP rats. Further, we have suggested that these changes could be related to GT's apoptosis-promoting ability. The current work has helped to define some of the cell types and cell functions that need further investigation as we seek to understand the immunomodulatory effects of GT.

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