Prophylactic fenbendazole therapy does not affect the incidence and onset of type 1 diabetes in non-obese diabetic mice

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Keywords: autoimmunity, diabetes, fenbendazole, prophylactic therapy

Abstract

Fenbendazole (FBZ) is a common, highly efficacious broad-spectrum anthelmintic drug used to treat and limit rodent pinworm infections. However, the effect of its prophylactic use on the immune response of rodents is largely undefined. The non-obese diabetic (NOD) mouse is a model commonly used to study type 1 diabetes (T1D). Parasitic infections will inhibit diabetes development in NOD mice; thus, in the presence of contamination, prophylactic treatment with anthelmintics must be considered to maintain experimental research. Herein, we investigated the prophylactic use of FBZ in NOD mice to determine its effect on the incidence and onset of diabetes, lymphocyte sub-populations and T cell proliferative responses. NOD mice were separated into control and treatment groups. The treatment group received a diet containing FBZ. Animals were monitored for the incidence and onset of T1D. At matched time points, diabetic and non-diabetic mice were killed and splenic lymphocytes analyzed for various cell sub-populations and mitogen-induced proliferative responses using flow cytometry. Treated and control mice were monitored >23 weeks with no detectable effects on the incidence or onset of diabetes. Moreover, no significant differences were detected in lymphocyte sub-populations and mitogen-induced CD4⁺ and CD8⁺ proliferative responses between control and treatment groups. These results suggest that prophylactic FBZ treatment does not significantly alter the incidence or onset of diabetes in NOD mice. The prophylactic use of FBZ, therefore, presents a viable approach for the prevention of pinworm infection in precious experimental animals with substantial scientific and economic benefits.

Introduction

Oxyurid parasites (pinworms), especially Syphacia obvelata and Aspiculuris tetraptera, are common contaminants of laboratory mice. Pinworm infections result in mild pathology, and unlike other common infections of laboratory rodents, are treatable with anthelmintic drugs (1, 2). However, it is unknown whether the use of these drugs for the treatment and/or prophylaxis of pinworms in rodents are associated with immune alterations, particularly in rodents prone to auto-immune complications.

Reportedly, fenbendazole (FBZ) is a safe, highly efficacious broad-spectrum anthelmintic drug with ovicidal, larvicidal and adulticidal actions. FBZ is commonly used in research institutions to treat rodent parasitic infections, notably pinworms, as well as prevent transmission to other colonies housed within the same facility. Studies using FBZ to treat and limit Syphacia muris infection in rats have shown that its action is both efficient and sufficient for decontamination (3–5). Large animal studies in cattle, sheep, pigs and goats indicate that the drug is oxidatively metabolized to an active compound oxfendazole sulfoxide and a less active sulfone metabolite (6–9). As a benzimidazole derivative, FBZ administered orally, usually through incorporation into the diet, inhibits parasite energy metabolism by blocking absorption of glucose and further polymerization of tubulin into microtubules. The anthelmintic action of FBZ is manifested through its affinity to parasitic, rather than mammalian, tubules (6, 10–12). In spite of the wide use of this agent, studies investigating the effect of prophylactic use of FBZ on various immunological parameters in normal and genetically modified rodents have been limited (13, 14).
The non-obese diabetic (NOD) mouse is a commonly used, spontaneous model for the study of insulin-dependent type 1 diabetes (T1D). Autoaggressive T cells of T1 phenotype, responding to pancreatic islet-specific antigens, initiate the disease and lead to the destruction of insulin-producing β cells (15–18). Interestingly, it has been shown that parasitic infections will inhibit diabetes development in NOD by counterbalancing and controlling the pathogenic T1 cells responsible for islet destruction through induction of normal anti-parasitic T1,2 type responses (19–21). Thus, when faced with pinworm and other parasitic contaminations, investigators using this model system must consider prophylactic treatment with anthelmintic drugs, like FBZ, to maintain experimental research. In this study, we investigated the prophylactic use of FBZ in uninfected NOD mice to determine if this anthelmintic drug altered the incidence and onset of diabetes as well as examined if prophylactic treatment influenced the splenic lymphocyte sub-populations and T cell proliferative responses. To the best of our knowledge, this study is the first to estimate the effects of prophylactic FBZ therapy in NOD mice. These data suggest that FBZ does not impact splenic T and B lymphocyte subsets or the ability of CD4+ and CD8+ T cells to proliferate. More importantly, prolonged exposure of NOD mice to FBZ does not modulate onset, progression or the incidence of diabetes.

## Methods

### Mice and glucose monitoring

A single shipment of female NOD/Lt mice, aged 8 weeks, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and randomly separated into two groups, control and FBZ treated, for the determination of cumulative diabetes incidence. Uninfected mice were maintained under specific pathogen-free conditions according to standards and guidelines stipulated by the University of Louisville Institutional Animal Care and Use Committee. Urine glucose levels were monitored with Chemstrip uGK urine test sticks (Roche, Indianapolis, IN, USA) twice per week. Positive urine glucose tests were confirmed by the blood glucose measurement using Prestige Smart System (Home Diagnostics, Inc., Ft Lauderdale, FL, USA). Mice with blood glucose values of >250 mg dl⁻¹ on two consecutive occasions were considered diabetic. Diabetic and non-diabetic, control and treated, animals were killed at matched time points 17–19 weeks and 29–31 weeks for analyses.

### Diet

For 23 weeks, the control group received Rodent Diet 5010 (Purina Mills, St Louis, MO, USA) while the treated group received the 5010 companion diet medicated with FBZ (mg kg⁻¹ diet). Sterilized water and autoclaved chow were provided ad libitum.

### Infection control

During the study, naïve animals housed in the same rack and room were tested by perianal cellophane tape tests and cecal float tests for *Syphacia* and *Aspiculuris* species. All tests were performed in house and were confirmed negative.

### Lymphocyte phenotyping and proliferation assay

Spleens from diabetic and non-diabetic control and treated mice were harvested and processed. Erythrocytes from spleen cell suspensions were hemolyzed, washed twice and stained with fluorochrome-conjugated antibodies against various cell-surface markers. Antibodies used to characterize lymphocyte sub-populations were anti-mouse CD3-FITC, CD25-PE, CD8-PerCp and CD4– and CD19-allophycocyanin. Cells were analyzed by flow cytometry on a FACS Calibur using Cellquest software (BD Pharmingen and Immunocytometry Systems, San Jose, CA, USA). Expressed percentages were based on gating using forward and side scatter parameters, as well as CD3+ expression for T lymphocytes. Results were graphed according to weighted average calculations and expressed as average percent ± SD.

For proliferation assays, splenocyte suspensions were labeled with 2.5 μM of carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA), resuspended in complete MLR medium [Dulbecco’s modified Eagle medium, pH = 7.0, containing 5% fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 137 mM L-arginine HCl, 1.36 mM folic acid, 27 mM L-asparagine, 100 U ml⁻¹ penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and 25 μM 2-mercaptoethanol (Sigma, St Louis, MO, USA) and stimulated with 2.5 μg ml⁻¹ of Con A (Sigma) for 72 h in a 37°C, 5% CO₂ incubator. Proliferative responses of T lymphocytes to Con A stimulation were determined by flow cytometry by detecting CFSE fluorescence and dilution thereof in CD4+ and CD8+ gated T cells. Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA) and lymphocyte responses expressed as proliferation indexes (PIs) using Mod-Fit (Verity Software House, Inc., Turramurra, Australia).

### Statistical analysis

Treatment differences in diabetes incidence were assessed by Kaplan–Meier life table analysis using the Tarone–Ware test. Non-parametric Mann–Whitney U test was used for all sub-populations and proliferation comparisons between control and treated groups. Weighted average calculations were performed for lymphocyte sub-population analysis for proper comparison between diabetic and non-diabetic animals killed and analyzed at 17–19 weeks to those at 29–31 weeks of age. Differences were considered significant when P-values were <0.05. All statistical tests were performed using SPSS 12.0 for Windows (SPSS, Inc., Chicago, IL, USA).

## Results

**Prophylactic FBZ therapy does not alter diabetes incidence or development in NOD mice**

In order to investigate the effects of prophylactic FBZ therapy on the incidence and onset of T1D in NOD, a single shipment of NOD mice were separated into control and treatment groups. For 23 weeks, treated mice received a diet containing FBZ. As shown in Fig. 1, prophylactic FBZ therapy does not modulate the incidence or development of diabetes in NOD female mice. Although FBZ-treated mice (n = 13) had greater...
incidence of diabetes (85%) as compared with control mice (n = 7) (71%), this does not significantly differ from the cumulative incidence (78%) of diabetes in our naive NOD colony (Fig. 1, P = 0.84, Kaplan–Meier life table analysis).

Taken together, these data demonstrate that prophylactic FBZ therapy does not alter the onset and incidence of diabetes in NOD mice.

Prophylactic FBZ therapy does not affect splenic lymphocyte sub-populations in NOD mice

It has been reported that FBZ treatment influences lymphocyte sub-populations in healthy, uninfected mice (13). Therefore, in order to investigate the effect of prophylactic FBZ treatment in NOD, splenocytes were harvested from age-matched diabetic and non-diabetic animals of both control and treated groups, stained with fluorescent antibodies against cell-surface markers and analyzed by flow cytometry. Within the spleen, the percentage of CD3+CD4+ lymphocytes between control (n = 5, 55.68 ± 7.39%) and FBZ-treated (n = 6, 57.87 ± 6.04%) animals did not significantly differ (P = 0.86). Likewise, differences in splenic percentage of CD3+CD8+ between control (38.03 ± 9.18%) and FBZ-treated (32.54 ± 5.59%) animals were not detected (P = 0.72, Fig. 2A).

Activated T cells are the major culprit of diabetes in NOD. To determine whether FBZ treatment affects the activation of T cells in vivo, splenocytes from control and treated animals were stained with a fluorescent-conjugated antibody against CD25, the α-chain of the IL-2R, and analyzed by flow cytometry. No significant difference between the percentage of CD4+CD25+ within the CD4+ T cell population was observed between the control (16.65 ± 2.46%) and treated (13.99 ± 2.11%) groups (P = 0.14, Fig. 2A). In addition, despite an increased CD4+ to CD8+ T cell ratio in FBZ-treated animals, 1.78 ± 0.48 as compared with 1.46 ± 0.60 in controls, this increase was insignificant (P = 0.58). Likewise, T to B lymphocyte ratios in control and treated animals were 1.37 ± 0.46 and 1.37 ± 0.37, respectively, and did not significantly differ (P = 0.78, Fig. 2B). Taken together, these data demonstrate that prophylactic treatment with FBZ does not significantly affect splenic lymphocyte populations in NOD mice.

Prophylactic FBZ therapy does not affect mitogen-induced CD4+ and CD8+ proliferative responses in NOD mice

Since differentiation of T cells is closely related to proliferative capacity and immunomodulatory effects of FBZ on healthy, non-parasitized rodents and other large animals has previously been shown (13, 22–24), the ability of NOD CD4+ and CD8+ T lymphocytes to respond to Con A stimulation were examined. Responses were reported as PIs based on CFSE dilution after 72-h Con A stimulation. CD4+ and CD8+ T lymphocytes from diabetic and non-diabetic animals were not significantly different in their proliferative capacities (Fig. 3A and B). Although the PI of both CD4+ and CD8+ T cells are increased in FBZ-treated (4.14 ± 2.11 and 5.82 ± 2.70) as compared with control (2.54 ± 0.90 and 3.65 ± 1.80) animals, this increase is not considered significant (P = 0.10, P = 0.10, Fig. 3C). As a general trend, both control and treated diabetic
animals demonstrated decreased CD4+ and CD8+ PIs as compared with non-diabetic animals (data not shown).

Discussion

Parasitic infections, especially pinworm, are common contaminants in rodent colonies (1, 2). In large research institutions, where facilities house numerous strains of mice, breeding colonies and experimental animal model studies, parasitic contamination and subsequent spread of infection could be devastating and is a major concern. To contain the infection and prevent further spread within an animal facility, FBZ, a broad-spectrum anthelmintic, is commonly used as a prophylactic treatment modality. Even though parasitic infection alone can influence physiologic processes and immune status of an animal (25–27), it is unclear whether the prophylactic use of this drug in uninfected animals has any immunomodulatory effects.

To this end, we investigated the effect of long-term prophylactic use of FBZ on splenic lymphocyte populations, proliferative responses and onset and incidence of diabetes in NOD mice. The choice of NOD mouse stems from its susceptibility to immunomodulation by parasitic infections and spontaneous development of T1D mediated by lymphocytes. As such, NOD provides a sensitive model to assess the effect of prolonged prophylactic treatment with FBZ on the lymphocyte populations and their function in vivo vis-à-vis the onset and incidence of diabetes. We herein demonstrated for the first time that prophylactic treatment with FBZ in NOD mice did neither significantly alter various lymphocyte sub-populations nor did it affect the proliferative response of T cells to Con A. More importantly, prolonged prophylactic exposure of NOD mice to FBZ did not modulate the onset, progression or incidence of diabetes.

The influence of FBZ administration on the immune response of parasitized and non-parasitized animals has been investigated in a number of studies. In uninfected Balb/c mice, FBZ administration had no effect on the ability of mice to generate primary allo-specific and influenza-specific cytolytic, helper, and memory T cell or antibody responses (28). Additionally, FBZ treatment did not eliminate eosinophilic infiltration into the ovaries or eyes in mouse models of autoimmune ovarian disease and experimental autoimmune uveitis infected with pinworms. Furthermore, these mice mounted normal anti-parasitic T1,2 type responses (29), suggesting that FBZ treatment does not influence immune responses in normal or autoimmune-prone animals. In contrast to the aforementioned studies, treatment of healthy C57BL/6 mice with FBZ
has been shown to enhance T and B lymphoproliferative responses to mitogens Con A and LPS (13). This immunostimulatory effect of FBZ is supported by studies in helminth-infected swine and healthy lambs (22–24). Additionally, FBZ treatment has been reported to decrease the percentage of splenic CD4+ T cells in favor of CD8+ T cells in healthy uninfected C57BL/6 mice (13).

Although the exact source of these apparent discrepancies remains unknown, multiple variables need to be considered. In general, when treating with anthelmintic drugs like FBZ, the presence or absence of the contaminating parasite and subsequent burden of infection as well as the insulting helminth, roundworms (*Ascaris* and *Toxicara*) versus pinworms (*Syphacia* and *Aspiculuris*), may have differential effects on immune responses. Different methods of administration, oral drench versus food incorporation, duration of treatments and time points analyzed post-treatment must also be taken into account. Anthelmintic drug treatment in uninfected animals may also be problematic, especially for irreplaceable experimental animals, due to the fact that extraneous administration of drugs could alter measured experimental outcomes. Different mouse strains, like C57BL/6 versus Balb/c, and their predilection to T1,2 versus T1,1 type immune responses may also contribute to differential effects of FBZ treatment in published studies (13, 28). Furthermore, immunomodulatory properties of FBZ may be affected by associated toxicities, particularly in genetically modified strains of mice and commonly used animal models of autoimmunity. Studies investigating the immunologic consequences due to the prophylactic use of FBZ in these model systems have not been well studied. However one study, evaluating the potential toxicity of prophylactic FBZ therapy in multiple genetically modified strains of mice reported a 16-week (8 weeks, 2 cycle) prophylactic FBZ treatment protocol, where FBZ was administered through incorporation into the rodent diet, had a low risk of toxicity (14).

In this study, we have shown that prolonged administration of FBZ to uninfected NOD mice did not affect lymphocyte subpopulations or T lymphocyte proliferative responses to Con A. The splenic percentage of CD4+ was greater than CD8+ and lymphocyte ratios (CD4+/CD8+ and CD3+/CD19+) were not significantly different between control and FBZ-treated mice. Furthermore, CD4+ and CD8+ T lymphocyte proliferative responses from diabetic and non-diabetic NOD mice treated with FBZ were not significantly different from control mice. These data are consistent with our observations that prophylactic FBZ treatment did not alter the incidence or onset of diabetes. Our results are also consistent with previous studies in normal and other autoimmune-prone animals, in which FBZ treatment did not affect specific cytolytic, helper and memory T cell or B cell antibody responses, inhibit normal anti-parasitic T1,2 type responses or prevent eosinophilic migration or infiltration in other autoimmune models (28, 29).

In contrast to our findings, immunostimulatory effect of FBZ has been reported in larger animals (22–24) and reportedly in healthy, uninfected C57BL/6 mice, FBZ inhibited T lymphocyte percentages, increased percent occurrence of splenic CD8+ T cells and stimulated T and B lymphocyte proliferative responses to polyclonal activators like Con A and LPS (13). Despite conflicting observations with our findings and as in any studies where significant differences are not observed, discrepancies to previously reported effects may be attributed to differences in mouse models/strains, FBZ dosage and treatment regimen, sample size, as well as time of analyses.

Taken together, our data suggest that prolonged prophylactic treatment of NOD mice with FBZ has no significant immunological consequences as assessed by the analysis of various lymphocyte populations, mitogen-stimulated proliferative responses and onset and incidence of diabetes. Although this study demonstrates the safe, effective use of FBZ in NOD mice as prophylaxis for parasite contamination, therapy and treatment in genetically altered strains of mice and other experimental animal models of disease should be studied and approached with caution.

Acknowledgements
This research was supported by grants from National Institutes of Health DK61333, AI47864, AI57903, Juvenile Diabetes Research Foundation (1-2001-328) and the Commonwealth of Kentucky Research Challenge Trust Fund. We would like to acknowledge Doug Lorenz in the Department of Bioinformatics and Biostatistics for assistance and critical review of the study data and statistics, as well as the manuscript. Additionally, we would like to thank Theresa Perry for initial statistical assessment, Brooke Parke, Lisa Colbert and Kathy Laster for their technical support and maintenance of our animal colony and Chantale Lacelle for critical reading of the manuscript.

Abbreviations
CFSE carboxy-fluorescein diacetate succinimidyl ester
FBZ fenbendazole
NOD non-obese diabetic
PI proliferation index
T1D type 1 diabetes

References