Comparative Medicine
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Vol 57, No 5
October 2007
Pages 487–492

Fenbendazole Treatment May Influence Lipopolysaccharide Effects in Rat Brain

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In evaluating discrepant results between experiments in our laboratory, we collected data that challenge the notion that anthelminthic drugs like FBZ do not alter inflammatory responses. We found that FBZ significantly modulates inflammation in F344 rats intrastriatally injected with LPS. FBZ treatment of LPS-injected rats significantly increased weight loss, microglial activation, and dopamine loss; in addition, FBZ attenuated the LPS-induced loss of astrocytes. Therefore, FBZ treatment altered the effects of LPS injection. Caution should be used in interpreting data collected from rats treated with LPS and FBZ.

Abbreviations: FBZ, fenbendazole; GFAP, glial fibrillary acidic protein; LPS, lipopolysaccharide

Pinworms are common parasites that compromise the health status of rodent colonies and cause numerous biologic alterations, such as elicitation of an immune response, growth modifications, and behavioral changes.[43,44,48,53,55,65] Therefore, rodent colonies are often treated for pinworms with anthelminthic drugs like fenbendazole (FBZ), ivermectin, dichlorvos, or permethrin.[6,15,16,19,24,39,56,59,64] However, several anthelminthic drugs also exert a variety of adverse side effects.[19,39,56,59,64]

Some of the most widely used anthelminthics belong to the benzimidazole group,[12,25] and their primary mechanism of action involves a high affinity interaction with nematode β tubulin, resulting in interference with microtubule assembly and disassembly.[37,53] However, benzimidazoles also bind mammalian tubulin[37] and influence the mitotic activity of rat cells.[11] This effect disturbs the equilibrium between tubulin and microtubule assembly and affects a wide range of cellular activities and organ functions.[25] The most commonly used benzimidazole, FBZ, has adulticidal, larvicidal, and ovicidal actions.[29,34,38]

This study originates from an attempt to replicate previous experimental results in our laboratory and to increase sample size in an experimental series. The original study was designed to assess possible synergism between a common industrial solvent, trichloroethylene, and a bacterial endotoxin, lipopolysaccharide (LPS), on degeneration of dopaminergic neurons in the substantia nigra, with the goal of producing a novel model of Parkinson disease. However, during the course of our studies, a parasitic infestation of *Aspiculuris tetraptera* occurred in the animal facilities at our institution. The infestation was diagnosed via the inhouse rodent health monitoring program and was found to affect 3 mouse rooms in the same facility that housed our rat colony. Although the room housing the rats in this study was negative for pinworms, given the number of rooms affected, distribution of the affected rooms, and practice of transferring mice and rats between multiple facilities at our institution for the use of shared research equipment and collaborative studies, a decision was made to treat the entire rat and mouse population with FBZ—a common practice for purging rodent colonies of pinworm infestations.[29,34,38]

Some of the rationale for using FBZ is as follows: (1) it is efficacious in treating *A. tetraptera* and *Syphacia muris* in mice and rats,[10,15,31] because it provides ovicidal, larvicidal, and adulticidal effects;[50] (2) FBZ has no known teratogenicity;[20] (3) it has a large margin of safety, with an acute oral LD_{50} of more than 10 g/kg in mice and rats, and toxicity does not occur in rats until reaching 60 times the therapeutic dose;[58,67] (4) FBZ has minimal behavioral effects and is deemed safe at therapeutic doses given for extremely long periods,[4] so behavioral experiments will not be affected; (5) it is considered to not effect the immune response,[52] so inflammation studies should not be changed or altered; (6) FBZ has comparatively less documented interference with research when compared with other antiparasitics such as ivermectin;[19,39,56,59] and (7) FBZ has low bioavailability in tissues.[40,41,49]

The current retrospective study was undertaken due to several unexpected results in rats that received both LPS and FBZ. Combined treatment with LPS and FBZ treatment caused a marked weight loss—an effect not noted previously in rats treated with this low dose of LPS.[32] The study was repeated using rats that were not being treated with FBZ. The second study showed no significant weight loss or mortality. We therefore compared the data from the saline-only and LPS-treated rats in the 2 studies (that is, with and without FBZ). We also analyzed microglial activation, astrocyte loss, and striatal dopamine in 18 rats that were treated with LPS+FBZ and 12 that received LPS only.

Materials and Methods

Animals. We obtained 5-mo-old male F344 rats from Harlan (Indianapolis, IN) and housed them under a 12:12-h light:dark cycle with free access to food and water at the University of Kentucky (Lexington, KY). All procedures were conducted in the Laboratory Animal Facilities of the University of Kentucky, which are fully accredited by the Association for Assessment and Accreditation of Laboratory Animals Care International. Experimental protocols involving the animals were followed in strict accordance

Received: 17 Apr 2007. Revision requested: 19 May 2007. Accepted: 21 Jun 2007.
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with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*[45] and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Treatments and injections. The rats were allocated randomly into treatment groups and given vehicle (olive oil) or 1000 mg/kg trichloroethylene (Sigma-Aldrich, St Louis, MO) by gavage on Monday to Friday for 1 wk prior to their injection with either saline or LPS. The LPS was used to elicit a low-grade innate immune response by administering 25% of a dose that used to elicit dopaminergic neurodegeneration.[33] In our previous unpublished studies, this dose had little detrimental effect on the rat nigrostriatal system; our hypothesis was that a low dose of LPS would enhance the negative effects of trichloroethylene alone.[32]

Intrastriatal LPS injections were performed described previously[33] with minor modifications. Rats were anesthetized with sodium pentobarbital (Abbott Laboratories, Chicago, IL), positioned in a stereotaxic apparatus, and received local anesthesia of 1:2000 bupivacaine with epinephrine (Hospira, Lake Forest, IL). Eight small openings were created in the skull by using a dental trephine. The stereotaxic coordinates, measured in millimeters from bregma, were: anterior–posterior, +1.0; medial–lateral, ±2.0 and ±3.5; and dorsal/ventral –5.5 and –6.0, as well as anterior–posterior –0.5, medial–lateral ±2.5 and ±4.0, and dorsal–ventral –5.0 and –6.5.[47] Next, 2 µl of either sterile saline or *Salmonella minnesota* LPS (1 µg/µl; Sigma-Aldrich) was injected into each site by using a 30-gauge, 10-µl Hamilton syringe. To ensure there was no error in formulating the LPS solution and that all animals received the same dose, the same lot number of LPS was dissolved and frozen in aliquots, with individual aliquots thawed for injection as needed. The rate of injection was 0.5 µl/min, and the needle remained in place for 5 min after injection before slow withdrawal.

After surgery, the rats were kept on a heating pad, and subcutaneous sterile saline was given to aid in postoperative recovery. In addition, a pain score rating[57] was used to ensure establishment of humane endpoints. Oral gavage continued for 5 wk after intrastriatal injection, and the rats were kept for 2 more weeks after gavage before euthanasia for data analysis. In rats treated with FBZ, LPS injections took place 5 to 10 d before the start of FBZ treatment, which continued until the rats were euthanized.

Weights. To monitor health status, body weight was recorded before the first gavaging, just before surgery, immediately after surgery, daily for the first week after LPS injections, and then weekly until euthanasia for tissue collection.

Colony health surveillance. The institution's rodent health monitoring program consists of the following: every 3 mo, sentinel mice and rats are placed (1 to a rack) in each room for 6 wk. During this time, they receive dirty bedding from every cage in the rack when cages in the room are changed, which occurs at least once weekly. After 6 wk, 1 rack sentinel from each room is sent to an outside laboratory (University of Missouri Research Animal Diagnostic Laboratory, Columbia, MO) for comprehensive serology, necropsy, parasitology, and microbiology tailored for each species, whereas the remaining sentinels are tested serologically by the same laboratory for an abbreviated panel of species-specific pathogens. In addition, inhouse testing for parasites is performed on these sentinels by means of tape testing of the shoulders and perineum and by direct examination of cecal contents. Identification of pinworm infestation would lead to a decision to treat the entire rat and mouse population with FBZ–a common practice used to purge rodent colonies of pinworm infestations.[29,34,38]

FBZ treatment. Once pinworm infestation was identified, the treatment schedule comprised 5 wk of FBZ-containing feed followed by 8 wk off treatment; this scheme was repeated for a total of 4 rounds. The duration of feed administration and use of multiple treatment periods reflects data from a survey of the current literature and previously successful treatment (involving a similar administration schedule) at the university to eliminate the pinworm *Syphacia muris* from mouse and rat colonies. During the course of treatment, at the cessation of each treatment cycle, random Sprague–Dawley (Crl:SD) rats and NU/NU (Crl:NU-*Foxn*1^{nu}) mouse sentinels were placed in each housing room and screened for *A. tetraptera* by cecal content examination 5 and 7 wk after exposure to colony animals via dirty bedding transfer. FBZ is administered as part of the rodents' diet in a pellet formulation containing the drug at 150 parts per million (TD01432, Harlan Tekland, Madison, WI), which provides a dose of 8 to 12 mg/kg body weight daily.

Immunocytochemistry. Immunocytochemistry was used to immunostain activated microglia by using the OX6 antibody (Serotec, Raleigh, NC) according to previously described procedures[3,33] or to label astrocytes by using the glial fibrillary acidic protein (GFAP) antibody (Cell Signaling, Danvers, MA) by using an avidin–biotin immunoperoxidase method.[42] Briefly, 30-µm sections from the substantia nigra were incubated overnight with primary antiserum at 4 °C. After washing and incubation with appropriate secondary antibodies (1:4000, Vector Laboratories), immunoreactive cells were visualized by using the avidin–biotin immunoperoxidase method (ABC Kit, Vector Labs, Burlingame, CA) with the chromagen 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). The number of OX6- or GFAP-positive cells within the outlines of the substantia nigra compacta region was determined by using the optical fractionator method of the Bioquant Image Analysis system (R & M Biometrics, Nashville, TN), which estimates the total number of activated microglia or astrocytes within the substantia nigra. The number of OX6- or GFAP-positive cells in the control rats was used to calculate the percentage of activated microglia or astrocytes in the LPS-injected rats. The cell counts were obtained by a researcher who was blinded to the treatment groups.

Optical fractionator method. Bioquant Image Analysis software (R&M Biometrics, Nashville, TN) was used to estimate the total cell number of activated microglia or astrocytes in the substantia nigra according to the following equation:[66]

$$N = \sum Q \times \frac{t}{h} \times \frac{1}{asf} \times \frac{1}{ssf}$$

where N is the total number of cells, ΣQ is the total number of cells counted per region of interest, t is the measured section thickness (30 µm), h is the height of the dissector (20 µm), 1/asf is the counting grid area (100 µm × 100 µm) divided by the dissector area (20 µm × 20 µm), and ssf is the sampling section fraction. Coefficients of error and variation were calculated to determine intra- and interanimal variation.

High-performance liquid chromatography. By using high-performance liquid chromatography as previously described,[14,33] tissue levels of dopamine and its primary metabolites, 3,4-dihydroxyphenylacetic acid and homovanillic acid, were measured, as were serotonin and its primary metabolite, 5-hydroxyindole acetic acid. Retention times of standards were used to identify peaks, and peak heights were used to calculate recovery of the internal standard (dihydroxybenzylamine) and the amount of

Table 1. Effect of treatment with LPS and FBZ on body weight of rats

Treatment	Baseline weight (g)	SEM	Weight at euthanasia (g)	SEM	% change	SEM
Saline+FBZ	333	10.22	344	12.12	3.94	1.61
LPS+FBZ	331	8.33	311	4.00	–5.04	0.21
Saline alone	333	7.57	333	7.05	1.56	1.61
LPS alone	341	8.58	344	10.91	0.25	0.98

SEM, standard error of the mean.
The percentage weight change data reveal that rats treated with both LPS and FBZ have significant ($P \leq 0.05$) weight loss.

monoamines and metabolites.

Statistical analysis. Data are expressed as mean ± standard error of the mean. Tests of variance homogeneity, normality, and distribution were performed to ensure that the assumptions required for standard parametric analysis of variance were satisfied. For weight, OX6, and GFAP data, GraphPad Prism software (version 4.03, GraphPad Software, San Diego, CA) was used to perform analysis of variance followed by the Newman–Keuls multiple-comparison post hoc test. First, chromatography data were analyzed from the 2 separate experiments by using unpaired *t* tests. LPS+FBZ-induced a significant decrease in striatal dopamine levels, but no dopamine loss was noted when compared with data from rats treated with LPS alone or saline alone. Next, because we wanted to analyze the effect of FBZ on LPS injection and to avoid any possible confounding variables, the LPS data were normalized to their respective saline injection controls. This normalization provided values that were percentages of their respective control value, and the normalized values were analyzed by using unpaired *t* tests. Statistical significance was defined as a *P* value of less than 0.05.

Results

LPS+FBZ induces significant weight loss. No significant difference was found among baseline weights. FBZ treatment caused no significant difference in weight changes after surgery in saline-injected rats, and both FBZ-treated and non-FBZ-treated rats gained weight after saline injections. However, by the end of the study, rats treated with both FBZ and LPS experienced significant (F value = 4.858; DF = 21; $P < 0.05$) weight loss (approximately 5%) when compared with all other groups (Table 1).

FBZ prolongs LPS-induced microglial activation. Immunostaining and unbiased stereologic cell counts of OX6-positive microglia revealed more (F value = 27.68; DF = 15; $P \leq 0.001$) positive cells in rats that received both LPS and FBZ (Figure 1, A).

LPS+FBZ induced loss of striatal dopamine. In an independent analysis of chromotography data from the 2 separate experiments, unpaired *t* tests showed that LPS+FBZ induced a significant ($P < 0.05$) decrease in striatal dopamine levels. However, no dopamine loss was apparent between rats treated with LPS alone and those given saline alone. However, when we compared the saline-injected controls of the 2 experiments, we observed a significant ($P < 0.01$) difference in the levels of dopamine. However, because we wanted to analyze the effect of FBZ on LPS injection, we normalized the LPS data to the respective saline-injected controls by making the LPS values percentages of the control values. After normalization, rats that received LPS+FBZ had significantly ($P < 0.05$) lower striatal dopamine levels than did rats given LPS alone (Table 2).

FBZ attenuated LPS-induced loss of GFAP-positive astrocytes. LPS alone induced a significant (F value = 7.779; DF = 15; $P < 0.05$) loss of GFAP-positive cells within the substantia nigra (Figure 1, B). However, rats treated with LPS+FBZ had no loss in the number of GFAP-positive cells, compared with those in either group of saline-injected rats (Figure 1, B).

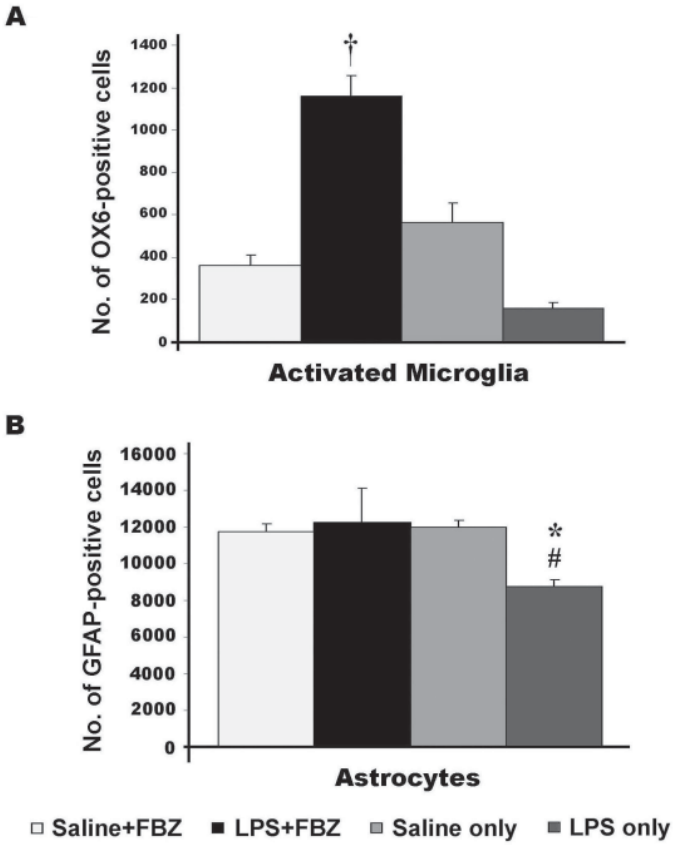

Figure 1. (A) Unbiased stereologic cell counts revealed significant differences in the number of OX6-positive microglia, where LPS+FBZ induced significant (†, $P \leq 0.001$) microglial activation compared with all groups. (B) Unbiased stereologic cell counts reveal that LPS induced a significant decrease in the number of GFAP-positive astrocytes, compared with all other treatments. #, $P \leq 0.01$ when the LPS alone group was compared with either saline-injected group; *, $P \leq 0.05$ when the LPS alone group was compared with the LPS+FBZ group.

Discussion

Pinworms are a common threat to rodent colonies, and infestations are often treated with FBZ.[29,34,38] However, our recent findings and a review of published data led us to hypothesize that FBZ may have immunomodulatory effects. We found that rats given FBZ after LPS administration had weight loss and greater

Table 2. Effect of treatment with LPS and FBZ on striatal dopamine levels in rats

Treatment	Dopamine (ng/g)	SEM	% of control	SEM
Saline+FBZ	13531	793	not applicable	not applicable
LPS+FBZ	9288	513	69	3.8
Saline alone	9762	647	not applicable	not applicable
LPS alone	10390	580	106	5.6

SEM, standard error of the mean.
LPS+FBZ induced a significant ($P < 0.05$) decrease in striatal dopamine levels, but no dopamine loss was observed when the LPS alone group was compared with the saline alone group. Normalization of data as percentage of the respective control values revealed that LPS+FBZ significantly ($P < 0.05$) decreased striatal dopamine levels.

microglial activation. In addition, LPS injection followed by FBZ treatment was associated with reduced striatal dopamine levels. Numbers of GFAP-positive astrocytes were reduced after injection of LPS alone; however, rats that received both LPS and FBZ had similar numbers of GFAP-positive astrocyte cells as did rats that received saline or saline and FBZ.

A previous report suggesting that FBZ treatment does not alter the immune response[52] examined changes in T lymphocytes and the ability to induce serum antibodies to viral proteins. However, primary immune responses such as macrophage activation and antigen presentation were not assessed. One published review suggested that FBZ has immunomodulatory activity.[54] An additional study using mice showed that FBZ treatment increased the proliferation of T and B lymphocytes that had been stimulated with LPS or concanavalin A and increased macrophage superoxide production.[21] LPS activates microglia,[2,36] which are the resident immune cells of the brain. Microglia and infiltrating monocytes both stain for rat major histocompatibility complex class II molecules OX6.[9,18] Our data show greater numbers of OX6-positive microglia in LPS-injected rats treated with FBZ, suggesting that FBZ prolongs LPS-induced microglial activation.

The reduction in striatal dopamine levels, when compared with control levels and only in the FBZ+LPS group, implies that the combination of LPS injection and FBZ treatment damages dopaminergic neurons. This finding correlates well with studies showing that excessive LPS-induced inflammation causes the loss of dopaminergic neurons and striatal dopamine.[26,30,33] The LPS dose we used was 25% of that reported to elicit dopaminergic neurodegeneration[33] and should have had little detrimental effect on the rat's nigrostriatal system. This hypothesis was confirmed, when data from rats treated with LPS alone were compared with those from rats given saline alone, and the striatal dopamine levels we recorded were similar to those we previously published.[33] We therefore speculate that FBZ promoted the LPS-induced microglial response to cause neurotoxicity. An alternative interpretation could be proposed as we saw a significant difference among the saline-injected controls, which could imply that FBZ modulates the dopaminergic system. However, further studies need to be performed to validate this theory.

In a study using sheep, FBZ was reported to decrease antibody production and serum complement, but this immunosuppression was variable, and the reported effects may have been due to components of the immune response other than lymphocytes.[11] Other studies have shown decreased lymphocyte sensitization and increased susceptibility to infectious challenge in parasite-infected mice treated with FBZ.[5,60] However, the cited data do not differentiate the effects of parasite elimination from those of FBZ administration. In addition, FBZ is reported to decrease the primary and secondary humoral response in FBZ-drenched lambs.[46]

Our data showed that LPS injection decreased the number of GFAP-positive astrocytes and that subsequent FBZ treatment mitigated this decrease. LPS activates astrocytes as well as microglia, because both cell types express the LPS receptor CD14,[7,23] which enables their cellular response.[63] The effect of LPS on astrocyte loss correlates well with other studies showing intranigral LPS induces astrocyte loss,[17,27] but why FBZ attenuated the LPS-induced GFAP-positive cell loss remains unclear. In our study, FBZ appears to potentiate microglial activation but protects against LPS-induced astrocyte loss. This effect may result from the different signal pathways used in LPS activation of microglia and astrocytes. Although astrocytes are not considered typical immune cells, they do elicit a toll-like receptor 4 response to LPS.[35] Our data indicate that FBZ may protect astrocytes against LPS toxicity; however, the potential mechanisms are unknown.

The bioavailability of FBZ in the central nervous system is low.[40,41,49] FBZ is reported to have efficacy in the gastrointestinal tract and lungs, and residues can be found in fatty tissues, muscle, liver, and kidney as long as 14 d after treatment.[20] One study suggested that once parasitic larvae reach the brain and musculature, they are resistant to anthelminthic agents.[1] However, the literature suggests that larvae in the brain are susceptible to FBZ treatment[8,13,18,21,22,28,61,62] and that FBZ can penetrate the blood–brain barrier.[22] Therefore, the bioavailability of FBZ in the brain may be sufficient to interact with other drugs or toxins such as LPS.

As previously mentioned, we initiated FBZ treatment because *A. tetraptera* was identified in the animal facility. The rats used to generate the data reported here were not tested for pinworm infection. However, the distribution of the pinworm infection in the animal facility and the negative sentinel screens in the room housing our rats suggest that they were not likely to have had pinworm infection. Two major caveats regarding our conclusions are that the study was not repeated and that control (FBZ-untreated) and experimental (FBZ-treated) groups were not studied at the same time. However, our results are not due to an error in the formulation of LPS or variations between batches, because aliquots of the same batch of LPS were used for both groups of rats. In our study, FBZ treatment began between 5 and 10 d after the injection of saline or LPS. This variation in the start time of FBZ treatment arose due to differences in the surgery schedules for rats given saline or LPS injections. However, because saline and LPS injected rats were injected in parallel, a similar number of animals were injected each day and for each study. In addition, these animals were euthanized according to their injection date, such that they were all exposed to LPS for the same amount of time. Another point is that animals were injected with LPS

before receiving FBZ treatment, such that an immune reaction was already occurring when FBZ treatment was initiated. The presence of a continued immune response so long after LPS injection in FBZ-treated rats supports an immunomodulatory activity in which FBZ at least potentiates the immune response. In summary, we report that FBZ-treated F344 rats have increased weight loss after intrastriatal LPS injection. Rats injected with LPS and treated with FBZ have increased microglial activation as well as loss of striatal dopamine. These findings are important because dopaminergic neurons in the substantia nigra are sensitive to inflammation-induced neurodegeneration.[3,26,33,42,51,68] In addition, we showed that FBZ attenuated the LPS-induced loss of GFAP-positive astrocytes. Overall, our data support an effect of FBZ on the immune response. Therefore, caution is needed when interpreting studies from animals treated with FBZ. Providing FBZ to animals with concurrent inflammation may exacerbate the inflammatory response.

Acknowledgments

Mei Liu, Laura Peters, the Division of Lab Animal Resources, and the funding supplied by the National Institute on Aging and National Institute of Neurological Disorders and Stroke: AG017963 (to WAC), NS044157 (to GYB), and PO1 AG13494 and P50 NS39787 (to DMG).

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