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Efficacy of an Experimental Azithromycin Cream for Prophylaxis of Tick-Transmitted Lyme Disease Spirochete Infection in a Murine Model

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As an alternative to oral prophylaxis for the prevention of tick transmission of *Borrelia burgdorferi*, we tested antibiotic cream prophylactic formulations in a murine model of spirochete infection. A 4% preparation of doxycycline cream afforded no protection, but a single application of 4% azithromycin cream was 100% protective when applied directly to the tick bite site at the time of tick removal. Indeed, the azithromycin cream was 100% effective when applied at up to 3 days after tick removal and protected 74% of mice exposed to tick bite when applied at up to 2 weeks after tick removal. Azithromycin cream was also protective when applied at a site distal to the tick bite site, suggesting that it was having a systemic effect in addition to a local transdermal effect. Mice that were protected from tick-transmitted infection did not seroconvert and did not infect larval ticks on xenodiagnosis. Azithromycin cream formulations appear to hold promise for Lyme disease prophylaxis.

Lyme disease is the most commonly reported vector-borne disease in the United States, with >30,000 cases reported to CDC annually (1). In fact, since Lyme disease became reportable in 1992, the number of cases reported to CDC has increased dramatically despite attempts at the federal, state, and local level to prevent this epidemic (2). The Lyme disease spirochete, *Borrelia burgdorferi*, is transmitted principally by the nymphal stage of the blacklegged tick, *Ixodes scapularis*, from mid-May to mid-June along the Atlantic seaboard from northern Virginia to Maine, with a smaller focus in the North Central United States (3). Approximately 80 million people live in states in the eastern half of the United States that contain regions where Lyme disease is highly endemic.

The first line of defense against tick-borne disease is certainly education regarding the risk of disease and the personal protection measures that are available (4). Unfortunately, avoidance of tick-infested areas, wearing of protective clothing, and using repellents and frequent tick checks are practiced inconsistently by the public; overall, these strategies have not been shown to be effective in blunting the Lyme disease epidemic, except under carefully controlled circumstances (5, 6). Despite a plethora of tick control tools developed by researchers, including area-wide acaricides, host-targeted acaricides, landscape modification, and least-toxic biological agents (4), actual proof that tick control can lead to a reduction in the incidence of Lyme disease is limited to a few efforts at deer eradication on islands (7, 8, 9).

More-direct approaches to preventing Lyme disease are those targeting humans, including vaccines and prophylactic use of antibiotics for tick bite. A Lyme disease vaccine based on recombinant outer surface protein A (OspA) was tested and shown to be 76% effective (10). This vaccine was released onto the U.S. market in 1999. Despite high hopes for the use of this tool to prevent Lyme disease, it was withdrawn from the market in 2002, ostensibly for market-related reasons. A variety of scientific, market, and legal issues may have contributed to the vaccine's withdrawal (11, 12). Antibiotic prophylaxis for tick bite in regions of high Lyme disease endemicity may be cost-effective (13). In a large clinical trial, Nadelman et al. (14) found a single dose of 200 mg of oral doxycycline to be 87% effective in preventing erythema migrans during a 6-week period of observation. The confidence intervals around this observation, however, were quite large (25% to 98%). The frequency of use of oral antibiotic prophylaxis for tick bite in the United States is currently unknown.

In order to better understand the dynamics of antibiotic prophylaxis to prevent Lyme disease, various researchers have conducted numerous experiments using a murine model and tick-transmitted spirochetal infection. Antibiotic prophylaxis has been delivered to mice via oral gavage (15, 16, 17), an injectable slow release formulation (15, 16), or topical applications (18, 19, 20). In the current study, the efficacy of topical creams containing doxycycline was compared to that of creams containing azithromycin. For the efficacious formulation (azithromycin), the time when, post-tick feeding, the cream must be delivered for maximum benefit was determined. In addition, whether the action of the cream is solely transdermal or whether it includes systemic action against tick-transmitted spirochetes was evaluated.

MATERIALS AND METHODS

Tick and bacterial strains. Nymphal *I. scapularis* ticks infected with the B31 strain of *B. burgdorferi* were produced as previously described (21). The colony originated from female ticks collected in New Jersey and Connecticut. Animals used in these experiments were female CD-1 mice, 4 weeks of age, purchased from Charles River Laboratories (Wilmington, MA). A single nymphal *I. scapularis* tick (>2 months post-larval feeding) was placed into a capsule on the back of each mouse and allowed to attach and feed as previously described (22). Nymphal ticks were allowed to feed for 72 h (near repletion), at which point they were removed from the host. Feeding to repletion generally takes 72 to 96 h for nymphal *I. scapularis* in

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TABLE 1 Efficacy of topical antibiotic cream preparations for prophylaxis against tick-transmitted spirochetes[a]

Drug	No. of spirochete-positive mice/no. of mice exposed	% protection
Azithromycin	0/12	100[b]
Doxycycline	11/12	8
Control cream	11/11	NA[c]

[a] Mice were treated on the day of tick removal.
[b] $P < 0.0001$ compared to control by Fisher's exact test.
[c] NA, not applicable.

our murine model (23). Animals were handled according to approved protocols on file with the Centers for Disease Control and Prevention, Division of Vector-Borne Diseases Animal Care and Use Committee Protocol numbers 10-007 and 13-002.

PCR analysis. Nymphal ticks were tested for infection with *B. burgdorferi* by PCR as previously described (24). Briefly, after ticks were removed from the host, DNA was extracted and tested for the presence of *B. burgdorferi* using a primer set directed against the *fliD* gene of *B. burgdorferi*. The cutoff limit for this quantitative PCR (qPCR) test was 20 spirochete equivalents per sample. All mice fed upon by PCR-negative ticks were removed from the experiment. Similarly, exposed mice upon which nymphal ticks did not attach and feed were removed from the experiment.

***Borrelia* culturing.** Mice were tested for infection with *B. burgdorferi* as previously described (25). At 1 month post-nymphal tick removal, mice were sacrificed and ears, urinary bladder, and heart were cultured in Barbour-Stoenner-Kelly (BSK) medium. Cultures were examined by darkfield microscopy weekly for 4 weeks for the presence of live spirochetes. In addition to the culture experiments, a subset of mice was tested for serological reaction to *B. burgdorferi*. Sera from experimental mice were tested at a dilution of 1:500 for antibody to *B. burgdorferi* utilizing the Marblot strip test system (MarDX Diagnostic Inc., Carlsbad, CA) per the manufacturer's instructions with the following modification: alkaline phosphatase-labeled goat anti-mouse IgG plus IgM (H+L) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was utilized as the detection antibody at a dilution of 1:2,500. Mice were considered reactive if there were ≥5 visible bands on the blot, per the manufacturer's specifications.

Xenodiagnosis. To further confirm the infection status of mice treated with azithromycin cream, we exposed mice to infected nymphs for 72 h and then treated them with azithromycin or control cream immediately upon nymphal tick removal. At ca. 3 weeks after the nymphal feeding, xenodiagnostic larval ticks from our uninfected colony were allowed to feed on these test mice, as well as on naive controls. Larval xenodiagnostic ticks were tested for the presence of spirochetes by PCR at 10 days post-larval feeding as previously described (24).

Antibiotic cream. Antibiotic creams were prepared with the assistance of a local pharmacy (Good Day Pharmacy, Loveland, CO). A 4% azithromycin cream was prepared by taking 3 tablets (250 mg each) and crushing them in 18.75 gm of Lipoderm cream (PCCA, Houston, TX); a trace amount of ethoxy diglycol was added to help form a paste. A control (nonantibiotic) cream was made the same way but without the azithromycin tablets added. A 4% doxycycline cream was prepared by adding 0.6 gm of doxycycline hyclate to 14.4 gm of anhydrous pluronic lecithin organogel (PLO; PCCA, Houston, TX). The preparation was made by wetting the doxycycline with trace amounts of dimethyl sulfoxide (DMSO) before mixing in syringes with anhydrous PLO. A nonantibiotic control cream was made the same way without the doxycycline added. Both cream preparations were delivered to the mouse by taking 50 μl of cream into a syringe and expressing the cream onto the designated spot on the skin where the tick capsule had been placed. Mice had been shaved to glue capsules onto the spot where ticks fed. The cream was then spread with the tip of the syringe. A total of 2 mg of the antibiotic was delivered to each of the treated mice. All mice were treated on only one occasion; no multiple treatments were performed in the course of these experiments.

TABLE 2 Effect of timing of delivery of topical azithromycin on the efficacy of prophylaxis against tick-transmitted spirochetes

Day post-tick bite	No. of spirochete-positive mice/no. of mice exposed	% protection
0	0/12	100[a]
1	0/11	100[a]
2	0/10	100[a]
3	0/12	100[a]
4	3/12	74[a]
5	2/8	74[a]
7	2/8	74[a]
14	3/12	74[a]
Control	19/20	NA[b]

[a] $P < 0.0005$ compared to controls by Fisher's exact test.
[b] NA, not applicable.

Statistics. The percent protection provided by antibiotic applications was calculated as previously described (17). Briefly, the formula $[1 - R_t/R_c] \times 100$ was used to calculate percent protection where R_t = the proportion of the treatment group infected and R_c = the proportion of the control group infected. Fisher's exact test was used to measure statistical significance.

RESULTS

The first trial conducted was a direct comparison of the protection provided by the doxycycline cream to that provided by azithromycin cream (Table 1). Animals in this trial were treated with the test cream right at the time of tick removal, after the ticks had been attached for 72 h. In the group receiving azithromycin cream, 0/12 mice developed spirochetal infection, representing a protection level of 100%. In sharp contrast, 11/12 of the mice receiving doxycycline cream developed spirochetal infection, representing only 8% protection. In this trial, all 11 control mice exposed to cream without antibiotics developed spirochetal infection. In subsequent experiments, we decided to test the limits of the azithromycin cream's efficacy rather than try to come up with a doxycycline formulation that gave greater protection.

The question of when post-tick-removal azithromycin cream had to be delivered to have maximal benefit was evaluated. A group of mice was treated on the day of tick removal (considered day 0 for the purpose of this experiment) or on subsequent days for up to 2 weeks following tick removal. Azithromycin cream treatment was 100% protective if delivered on day 0, day 1, day 2, or day 3 following tick removal (Table 2). Although the level of protection fell off somewhat to 74% if delivered ≥4 days following tick removal, it was still at the 74%-protective level even at 2 weeks following tick removal.

The next series of experiments was aimed at determining whether the azithromycin cream had to be delivered directly to the site of the tick bite or could be delivered to the skin at a distal site. In experiments where a distal site was treated, mice were shaved on the belly and cream was delivered to this ventral location rather than to the dorsal location where the tick capsule had originally been placed. We also tested whether ingestion of the antibiotic cream during mouse grooming played a role in the efficacy of treatment. In these experiments, a capsule was left in place and the cream was placed inside the capsule where it could not be groomed off by the mice. The capsule was left for 24 h either at the tick bite site or the distal location on the ventral side of the mouse. It did not matter whether the treatments were placed at the tick

TABLE 3 Effect of treatment site and grooming on the efficacy of azithromycin cream treatments for prophylaxis against tick-transmitted spirochetes

Treatment site or treatment	Grooming allowed	No. of spirochete-positive mice/no. of mice exposed	% protection
Bite site	Yes	0/15	100[a]
Bite site	No	1/18	92[a]
Distal	Yes	0/10	100[a]
Distal	No	0/12	100[a]
Control cream	NA	7/10	NA[b]

[a] $P < 0.0001$ compared to controls by Fisher's exact test.
[b] NA, not applicable.

TABLE 4 Spirochetal infections in xenodiagnostic larval ticks fed on mice treated with antibiotic creams[a]

Treatment group	No. of mice	No. of spirochete-positive ticks/no. of ticks examined
Azithromycin	10	0/118[b]
Naive controls	4	0/39[b]
Control cream	4	47/47[b]

[a] A total of 8 to 12 ticks from each mouse were tested by PCR at 10 days postrepletion.
[b] $P < 0.0001$ compared to controls by Fisher's exact test.

bite site or the distal location (Table 3). Where grooming was allowed, 100% of mice treated at the tick bite site and 100% of mice treated at the distal location were protected. Where grooming was not allowed, 92% of mice were protected when treated at the tick bite site and 100% of mice were protected when treated at the distal location. In all, only 1 of 38 treated mice in this experiment was not protected from infection.

To confirm culture results, sera from 10 mice judged to be infected by culture and 27 mice judged to be negative by culture were selected from the overall data set and tested for evidence of antibodies to *B. burgdorferi* by MarBlot. All 10 mice judged to be positive via culture had a positive serological result, and all 27 mice judged to be negative via culture had a negative serological result (data not shown).

A total of 10 mice treated with azithromycin were subjected to feeding by xenodiagnostic larval ticks; 118 xenodiagnostic larvae derived from these mice were all negative for spirochete DNA upon PCR examination (Table 4). Similarly, 4 naive control mice produced a total of 39 ticks negative for spirochetes on PCR examination. In contrast, 47/47 (100%) larval ticks fed on 4 positive mice treated with control cream proved positive for spirochetal DNA on PCR examination.

DISCUSSION

Previous experiments with azithromycin have demonstrated that ≥4% topical creams can provide 100% protection when applied to mice twice daily for 3 days (19). The current work extends these observations and demonstrates that a single application of 4% azithromycin cream can be 100% protective when applied within the first 3 days following a tick bite in a murine model system. Interestingly, a commercially available 2% erythromycin topical ointment failed to protect mice against tick-transmitted infection with *B. burgdorferi* (20). At present, we do not know whether azithromycin is much more effective than the closely related macrolide antibiotic erythromycin or whether the difference in the concentration of the drug is a key element in their respective levels of success. Azithromycin has long been known to be more active than erythromycin against certain Gram-negative bacteria (26).

Our results with doxycycline prophylaxis in our murine model system have been mixed. Single-dose oral doxycycline given by gavage has resulted in disappointing levels of prophylaxis, demonstrating 20% to 43% protection (15, 16). In contrast, a slow-release injectable doxycycline gel that continues to release doxycycline into the bloodstream over several weeks gave 100% protection (15). Protection afforded by oral doxycycline in our murine model was increased to 74% only by delivering the drug twice daily on the day ticks were removed (17). Results with topical applications of doxycycline and tetracycline have been inconsistent. Shih and Spielman (18) found doxycycline to be completely protective but did not give details of the experiment. They found tetracycline to be protective only when delivered for 3 days compared to the ineffective results found when tetracycline was given for a single day. Wormser et al. (20) found a 3% tetracycline preparation to be ineffective in providing prophylaxis for tick-transmitted *B. burgdorferi* in a murine model. In addition, our experiments in the current study found single topical applications of 4% doxycycline to provide no protection against tick-transmitted infection with *B. burgdorferi*, even when delivered on the day of tick removal. The tendency of azithromycin to collect in tissues may offer an advantage over doxycycline when applied as a cream to skin. Overall, it appears that azithromycin holds more promise than doxycycline for development of cream preparations for prophylaxis for Lyme disease spirochetes.

The fact that azithromycin cream gave 100% protection when applied at any time during the first 3 days following tick removal, but continued to give high levels of protection (74%) even after 2 weeks post-tick removal, suggests that the action of the cream is both prophylactic and curative. The spirochete infection is well established in the rodent host by 2 weeks post-tick removal (27) and disseminates from the site of tick attachment by ≥3 days after spirochete transmission (28). Azithromycin must be curing well-established infections as well as preventing the infection from establishing in the first place. Experiments conducted in this study also support the suggestion that the action of the cream is transdermal (19) since mice that could not ingest the cream were protected. But, since cream placed distal to the site of tick feeding was also protective, the azithromycin must have been having a systemic effect in addition to a local effect. It is questionable whether the systemic effect of azithromycin cream seen in mice would be also observed in much larger hosts (such as humans) if the drug were to be applied topically to skin, since the drug would be diluted in the greater blood volume. Experiments with larger hosts might help determine whether the systemic effect of azithromycin for protection against Lyme disease spirochetes is essential or whether the transdermal effect suffices to give protection. Larger hosts from rabbits (29) to rhesus monkeys (30) have been used to study the dynamics of tick-transmitted Lyme disease spirochete infection. Finally, the fact that Knauer et al. (19) observed that the concentrations of azithromycin in the skin (mg/liter) was >100,000 × MIC of azithromycin for *B. burgdorferi* in dermal treatment sites suggests that spirochetes are being affected locally within the skin at the bite site.

A topical antibiotic cream could be prescribed to residents living in regions where Lyme disease is highly endemic in the begin-

ning of the nymphal *I. scapularis* activity season (mid-May). Residents could self-apply the cream right at the time of tick removal. An extremely small amount of antibiotic would be used, reducing worries about selecting for antibiotic-resistant bacteria. Steps along the way toward such a product would include additional animal model work in larger animals, the development of a topical formulation of azithromycin by a commercial entity, and, eventually, human clinical trials. Interestingly, according to a personal communication from Reinhard Straubinger, Munich, Germany, and Gustave Huber, Zürich, Switzerland, "Ixodes AG* has performed clinical trials up to phase III. Phase I and II studies demonstrated safety and efficacy (surrogate endpoint based on azithromycin concentrations in different skin layers exceeded published MIC for borrelia). The phase III trial confirmed excellent safety and showed a positive trend to prevent clinical signs of Lyme borreliosis. Since the number of evaluable cases was not sufficient to test the null hypothesis with a statistical significance, a second phase III trial is being planned." The outcome of that clinical trial should be of some public health interest.

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