A sustained-release formulation of doxycycline hyclate (Atridox) prevents simultaneous infection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* transmitted by tick bite

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Current prophylaxis for infected tick bites consists of personal protective measures directed towards ticks. This study compared the efficacy of a single oral dose of doxycycline with that of a single injection of sustained-release doxycycline in a model of Lyme borreliosis and *Anaplasma phagocytophilum* infection. Dosages of doxycycline were equilibrated based on previously determined peak plasma levels in mice [oral, 2.4 µg (ml plasma)⁻¹; sustained release, 1.9 µg (ml plasma)⁻¹] determined 8 h after inoculation. In challenge experiments where five *Borrelia burgdorferi*-infected and five *A. phagocytophilum*-infected nymphs were used per mouse, only 20 and 30 % of mice were protected from *B. burgdorferi* and *A. phagocytophilum* infection, respectively, using oral doxycycline. In contrast, 100 % of mice receiving sustained-release doxycycline were protected from *A. phagocytophilum* infection, as indicated by real-time PCR of blood samples, quantitative PCR and culture isolation of spleen samples, and protected against *B. burgdorferi* infection as demonstrated by culture of ear, heart and bladder. Although 15–40 copies of *A. phagocytophilum* could be amplified from the spleens of mice treated with sustained-release doxycycline, no viable *A. phagocytophilum* from these spleens could be cultured in HL-60 cells. In contrast, 7/10 mice receiving oral doxycycline were PCR- and culture-positive for *A. phagocytophilum*, with copy numbers ranging from 800 to 10 000 within the spleen, as determined by quantitative PCR. Other correlates with *A. phagocytophilum* infection included a significant difference in spleen mass (mean of 110 mg for sustained-release treatment versus a mean of 230 mg for oral treatment) and the number of splenic lymphoid nodules (mean of 8 for sustained-release treatment versus mean of 12.5 for oral doxycycline) as determined by histopathology. These studies indicate that a single injection of a sustained-release formulation antibiotic may offer a viable prophylactic treatment option for multiple infectious agents in patients presenting with tick bites.

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INTRODUCTION

Lyme borreliosis is the most common vector-borne disease reported in the USA (CDC, 2004). Human granulocytic anaplasmosis (formerly known as human granulocytic ehrlichiosis) is an emerging tick-borne disease first described in the midwestern USA, and now routinely found along the north-eastern seaboard and upper midwestern states (Bakken & Dumler, 2000; McQuiston *et al.*, 1999). The aetiological agent of human granulocytic anaplasmosis is

Anaplasma phagocytophilum and is transmitted in these parts of the USA by *Ixodes scapularis*, the same tick vector that transmits Lyme disease in these geographical regions (Massung *et al.*, 2004; Pancholi *et al.*, 1995). Both of these zoonotic pathogens are maintained in cycles comprising *I. scapularis* ticks and *Peromyscus leucopus* reservoir mice (Stafford *et al.*, 1999). Infection with one of these agents may accompany infection of the other, both in vector ticks and reservoir hosts (Adelson *et al.*, 2004; Stafford *et al.*, 1999). Moreover, within endemic areas, humans may acquire combined infections, leading to overlapping disease symptomatology (Aguero-Rosenfeld *et al.*, 2002; Krause *et al.*,

Abbreviation: CDC, Centers for Disease Control and Prevention.

2002). Clinical delineation of this becomes important, as acknowledging dual infection may affect the inclusion of appropriate and effective chemotherapy for *A. phagocytophilum*, which may be different from treatment for acute Lyme borreliosis (Maurin *et al.*, 2003; Nadelman *et al.*, 1997).

Doxycycline is the drug of choice for treating patients with acute *A. phagocytophilum* infection. Likewise, although amoxicillin is widely used to treat acute Lyme borreliosis, studies indicate that doxycycline may be as effective (Wormser *et al.*, 2003). Similarly, some success has been obtained using a single dose (200 mg) of doxycycline hyclate for prophylactic treatment of people exposed to an *I. scapularis* tick bite (Nadelman *et al.*, 2001). Our laboratory has reported that delivery of a single dose of a sustained-release formulation of doxycycline hyclate is 100% effective in preventing tick-transmitted *Borrelia burgdorferi* infection in a murine model of Lyme borreliosis (Zeidner *et al.*, 2004a). Moreover, similar results have been reported in a murine model of tick-transmitted *A. phagocytophilum* (Massung *et al.*, 2005). Compared with the rapid clearance of orally delivered doxycycline, sustained-release doxycycline plasma levels in these studies were sustained over a 19 day period after delivery at a concentration below the MIC reported for *B. burgdorferi* (Johnson *et al.*, 1990) and with no apparent toxicity. In general, controlled-release delivery systems for antimicrobial agents have been shown to increase the bioavailability of short-lived antibiotics to mammalian tissues and to enhance treatment efficacy (Matschke *et al.*, 2002).

In contrast to previous studies looking at treatment of a single infectious agent transmitted by ticks, the current studies were done to compare the effectiveness of a single oral administration of doxycycline hyclate with a single subcutaneous administration of sustained-release doxycycline to prevent dual transmission of *A. phagocytophilum* and *B. burgdorferi* simultaneously delivered by *I. scapularis* ticks.

METHODS

Tick transmission of *B. burgdorferi* and *A. phagocytophilum*.

Laboratory-reared, *B. burgdorferi*-infected *I. scapularis* nymphal ticks were raised as described by Piesman (1993) and have been shown previously to be free of *A. phagocytophilum* and *Babesia microti* (Zeidner *et al.*, 2004a). These ticks were infected with low-passage-number *B. burgdorferi* strain B31. Likewise, laboratory-reared nymphal *I. scapularis* ticks that had previously fed as larvae on *P. leucopus* mice infected with a low-passage-number of *A. phagocytophilum* Webster strain (Massung *et al.*, 2004) were used for tick inoculation studies. In dual-infection studies, a total of ten infected nymphs (five of each infected with *B. burgdorferi* or *A. phagocytophilum*) were placed on the head and neck areas of specific-pathogen-free, 6-week-old female C3H/HeJ mice ($n=5$ per group, mass equal to 20.1 ± 0.2 g; Jackson Laboratory). In single infection control studies, only five infected nymphs harbouring either *B. burgdorferi* or *A. phagocytophilum* were placed on mice. These studies were then repeated with $n=5$ mice per group. At 72 h after tick infestation, the partially engorged ticks were removed from all mice as described previously (Massung *et al.*, 2005; Zeidner *et al.*, 2004a). The mice were then randomly assigned to receive either 2 mg oral doxycycline

hyclate in water, 4.2 mg sustained-release doxycycline hyclate co-polymer formulation (Atridox) (QLT Laboratories), or water or DL-lactide in *N*-methyl-2-pyrrolidone co-polymer (QLT Laboratories) vehicle treatment controls. These dosages of doxycycline were previously shown to deliver equivalent peak levels of drug in mouse plasma measured 8 h after inoculation (Zeidner *et al.*, 2004a). Oral doxycycline was delivered by gavage in 0.1 ml tissue-grade water. The sustained-release doxycycline hyclate (Atridox) was mixed according to the manufacturer's specifications and transferred to a 1 ml Luer Lock syringe (Becton Dickinson) fitted with a 25-gauge needle for subcutaneous injection. A total of 0.05 ml was then delivered to each mouse along the dorsal midline of the animal between the scapulae.

Determination of *B. burgdorferi* and *A. phagocytophilum* infection in mice.

Mice were bled weekly for 3 weeks post-tick infestation to determine the *A. phagocytophilum* infection status in blood. These EDTA-treated blood samples were frozen at -80°C until analysed. At 3 weeks post-treatment, all mice were euthanized and skin (ear biopsy), heart and bladder were cultured in Barbour-Stenner-Kelly medium (Schwartz *et al.*, 1992) to determine the *B. burgdorferi* infection status (Sinsky & Piesman, 1989). Samples were also placed in tissue fixative (Streck Laboratories) to determine histopathology. Likewise, spleen samples were taken to determine *A. phagocytophilum* infection levels, both by PCR and by direct culture using HL-60 cells. All spleens were first weighed, and equal amounts of tissue were either frozen at -80°C until analysed by PCR, or homogenized and co-cultured on human promyelocytic HL-60 cells. The remainder of the spleen was then placed into tissue fixative for histopathological evaluation. Growth of *A. phagocytophilum* in HL-60 cells was monitored by light microscopy after methanol fixation of cytocentrifuge preparations and staining with the Diff-Quik diagnostic stain (Dade Behring).

DNA extraction and quantification of *A. phagocytophilum* copy number.

A real-time PCR assay (Massung *et al.*, 2004, 2005) was utilized to determine the presence of *A. phagocytophilum* in peripheral blood, and a quantitative PCR assay was used to determine *A. phagocytophilum* copy number in spleen samples. DNA was extracted from tissues as described previously (Massung *et al.*, 2004, 2005) using a DNeasy tissue kit (Qiagen). In the case of blood, 200 μl whole blood per mouse was used, and copy numbers in spleen were determined [copy number (mg spleen tissue) $^{-1}$]. The real-time PCR assay utilized primers and probe to amplify the spacer region between the single-copy 23S and 5S rRNA genes as described previously (Massung *et al.*, 2004). Real-time PCR assays were performed on each sample in triplicate and the mean was determined per sample. Both positive and negative controls were included in all PCR assays.

Determination of histopathology. Tissue sections were prepared as described previously (Zeidner *et al.*, 2004b). Briefly, specimens placed in Streck's tissue fixative were subjected to standard processing, embedded in paraffin and sectioned at 5 μm . Sections were then stained with haematoxylin and eosin for standard light microscopy evaluation. All tissue sections were read and analysed in a coded, blind fashion.

Statistical analysis. Fisher's test was utilized to determine significant differences among treatment groups. Student's *t*-test was used to determine significant differences in splenic copy numbers of *A. phagocytophilum* among treatment groups. In both cases, $P<0.05$ was considered a statistically significant difference between groups.

RESULTS

As shown in Table 1, sustained-release doxycycline hyclate completely protected mice (100%) from both *B. burgdorferi*

Table 1. Treatment efficacy of sustained-release doxycycline hyclate against *B. burgdorferi* co-transmitted with *A. phagocytophilum*

	No. of mice infected with Bb/no. tested	Protection (%)*	No. of mice infected with Ap/no. tested	Protection (%)†
Sustained-release doxycycline	0/10	100	0/10	100
DL-Lactide co-polymer control	7/10	NA	7/10	NA
Oral doxycycline	8/10	20	7/10	30
Water control	10/10	NA	8/10	NA

Ap, *A. phagocytophilum*; Bb, *B. burgdorferi*; NA, not applicable.

*Culture results of skin biopsy analysed at 28 days, and heart and bladder at 56 days post-tick infestation.

†Results of quantitative PCR analysis and culture (spleen) at 21 days post-tick infestation.

and *A. phagocytophilum* infection transmitted simultaneously by infected ticks. In comparison, 70 and 80 % of mice receiving a single dose of oral doxycycline became infected with either *A. phagocytophilum* or *B. burgdorferi*, respectively, which was not statistically different from either the co-polymer or water control mice ($P=0.2$). In the case of *B. burgdorferi*, infection status was based on culture of skin (ear), heart and bladder. Where animals were infected with *B. burgdorferi*, all three tissues were consistently infected and demonstrated pathology of the heart, spleen and bladder (data not shown). In the case of *A. phagocytophilum* infection, *A. phagocytophilum* DNA could not be detected within the peripheral blood on days 7, 14 and 21 in mice receiving sustained-release doxycycline, and that were infected with *A. phagocytophilum* alone or simultaneously challenged with *B. burgdorferi* (Fig. 1a). In contrast, DNA could be amplified from the blood of 7/10 mice receiving oral doxycycline on days 7, 14 and 21 (Fig. 1a). As previous studies have indicated that the spleen is a site of *A. phagocytophilum* replication and accumulation, we analysed the spleen on day 21 by quantitative, real-time PCR and by direct isolation of *A. phagocytophilum* from cultured HL-60 cells. As shown in Fig. 1(b), mice challenged with *A. phagocytophilum*-infected ticks alone or simultaneously with *B. burgdorferi*-infected ticks, and treated with Atridox, demonstrated mean values of 21.6 and 15.3 copies (mg spleen^{-1}), respectively, as measured by quantitative PCR. These copy numbers were significantly different from those in mice treated with oral doxycycline (dual infection, mean of 987 copies mg^{-1} , $P<0.001$; *A. phagocytophilum* infection alone, mean of 3892 copies mg^{-1} , $P<0.001$). Moreover, there was no statistically significant difference in *A. phagocytophilum* splenic copy number between mice treated with oral doxycycline and the co-polymer or water control (dual infection, mean of 987 versus 1181 copies mg^{-1} , $P=0.76$; *A. phagocytophilum* infection alone, mean of 3892 versus 768 copies mg^{-1} , $P=0.24$). Attempts to culture *A. phagocytophilum* from spleen homogenates from mice treated with sustained-release doxycycline hyclate showed no growth, suggesting that the PCR-positive spleens from these mice did not contain viable organisms (data not shown).

Previous studies have indicated that replication and accumulation of *A. phagocytophilum* occurs within the

spleen (Massung *et al.*, 2004, 2005). As shown in Fig. 2, a marked difference in the size of spleens was noted when comparing mice treated with oral doxycycline with those

Fig. 1. (a) PCR amplification of *A. phagocytophilum* from the blood of tick-infested mice. Blood samples were harvested on days 7, 14 and 21 post-tick infestation for quantitative real-time PCR analysis as described in Methods. Points represent the mean values per treatment group ($n=10$) of mice. (b) Representative copy numbers of *A. phagocytophilum* PCR-amplified from the spleen of mice 21 days after tick infestation. Lines represent the mean copy number value per treatment group. Ap, *A. phagocytophilum*; Bb, *B. burgdorferi*.

Fig. 2. Histopathology following dual *B. burgdorferi* and *A. phagocytophilum* challenge. (a) A representative spleen sample (magnification $\times 5$) of an animal treated with sustained-release doxycycline hyclate (Atridox). Arrows indicate discrete lymphoid follicles. (b) A representative tissue sample (magnification $\times 5$) of an animal treated with oral doxycycline, which was both PCR- and culture-positive for *A. phagocytophilum*, as well as culture-positive for *B. burgdorferi*.

treated with Atridox. Marked lymphoid hyperplasia and an increase in cellular infiltrate (monocytes, neutrophils and plasma cells) within the red pulp were noted for animals treated with oral doxycycline who were both PCR- and culture-positive for *A. phagocytophilum* (Fig. 2b). In contrast, those animals in which *A. phagocytophilum* and *B. burgdorferi* infection was prevented retained a normal splenic architecture with discrete lymphoid follicles and no extraneous cellular infiltrate within the red pulp (Fig. 2a). Moreover, gross spleen mass was significantly different when comparing treatment groups ($n=10$). Those animals in which infection was prevented by treatment with Atridox demonstrated spleen masses ranging from 98.6 (dual infection) to 121 mg (*A. phagocytophilum* infection alone) similar to normal ($n=10$) C3H/HeJ spleens (mean of 107 ± 0.2 mg, $P=0.28$) (Fig. 3a). In contrast, animals treated with oral doxycycline had spleen masses ranging from 213 to 244 mg (Fig. 3a), which was statistically significant ($P<0.0005$). Moreover, there was no difference in spleen masses of animals treated with oral doxycycline compared with the water- or co-polymer-treated controls ($P=0.51$). We noted that the increase in spleen mass could be due to the marked lymphoid hyperplasia in PCR-positive spleens, as well as the number of lymphoid nodules enumerated (Fig. 3b). In Atridox-treated mice, which resisted infection, the mean number of lymphoid nodules was 7.6, equivalent to the number of nodules found in normal C3H/HeJ mice (mean of 7.0 ± 0.3 , $P=0.51$) (Fig. 3b). In contrast, in animals treated with oral doxycycline or vehicle treatment controls, which did not resist infection, the average number of lymphoid nodules ranged from 11.9 to 12.6 ($P<0.001$). As with spleen mass, there was no statistically significant difference between animals treated with oral doxycycline and those receiving vehicle controls (Fig. 3b), whether water or co-polymer ($P=0.13$).

DISCUSSION

Previous studies in our laboratory have demonstrated that a single injection of a sustained-release form of doxycycline hyclate completely protects mice from tick-transmitted *B. burgdorferi* infection (Zeidner *et al.*, 2004a). A second study in our laboratory indicated 100% protection against tick-transmitted *A. phagocytophilum* infection in a similar

Fig. 3. (a) Comparative spleen masses of mice following tick infestation. The bars represent the mean mass per treatment group $\pm$ SD ($n=10$). **, $P<0.05$ between the Atridox-treated group and the co-polymer- or water-treated vehicle control mice. (b) Quantitative histopathology of spleens 21 days after tick infestation. The bars represent the mean number of lymphoid nodules per treatment group ($n=10$). **, $P<0.05$ between the Atridox-treated groups and the co-polymer- or water-treated vehicle control mice. Ap, *A. phagocytophilum*; Bb, *B. burgdorferi*.

mouse model (Massung *et al.*, 2005). Our previous studies indicated that oral delivery of 2 mg doxycycline hyclate to mice gave peak levels of $2.43 \mu\text{g doxycycline (ml plasma)}^{-1}$ at 8 h after delivery, slightly higher than the peak levels in mice given 4.2 mg sustained-release doxycycline [$1.89 \mu\text{g doxycycline (ml plasma)}^{-1}$] (Zeidner *et al.*, 2004a). The present study was undertaken to determine whether a single injection of sustained-release doxycycline hyclate would be effective at preventing both *B. burgdorferi* and *A. phagocytophilum* delivered simultaneously by *I. scapularis* ticks. Although several *in vitro* studies have indicated that *A. phagocytophilum* is highly sensitive to doxycycline (Horowitz *et al.*, 2001; Maurin *et al.*, 2003), our study is the first to demonstrate that a sustained-release formulation of doxycycline is completely effective against simultaneous delivery of *B. burgdorferi* and *A. phagocytophilum* by questing ticks *in vivo*. In these studies, a single dose of oral doxycycline prevented *B. burgdorferi* infection in only 20 % of the animals tested (Table 1), which was not significantly different from animals given a copolymer or a water vehicle treatment control. These results are in contrast to the 43 % protection achieved in earlier studies against *B. burgdorferi* (Zeidner *et al.*, 2004a), possibly due to the increased number of ticks placed on each animal ($n=10$) to achieve dual infection. Although not analysed in this study, tick saliva has been shown previously to enhance both viral and bacterial transmission *in vivo* (Nuttall & Labuda, 2004; Zeidner *et al.*, 2002), and this effect may be limiting the effectiveness of this dose of oral doxycycline. Moreover, we do not know from these studies whether the simultaneous delivery of both *B. burgdorferi* and *A. phagocytophilum* had any effect on the subsequent metabolism of doxycycline hyclate administered orally.

Quantitative real-time PCR data (Fig. 1) indicated that, in productive *A. phagocytophilum* infection (both PCR- and culture-positive spleen), the copy number for *A. phagocytophilum* ranged from 800 to 10 000 copies within the spleen (Fig. 1b). Moreover, these studies indicated that a threshold value of 100 copies of *A. phagocytophilum* in the spleen may be associated with productive infection *in vivo*. In animals that were below this threshold copy number (Fig. 1b), DNA could not be detected in the peripheral blood, and spleens were subsequently culture-negative when co-cultured on HL-60 cells. Likewise, no splenomegaly, lymphoid hyperplasia or splenitis (Fig. 2) was noted in those animals that were successfully treated and resisted tick-transmitted infection. In these studies, splenomegaly and lymphoid hyperplasia correlated with treatment failure (Fig. 3). The gross mass of the spleens of animals receiving either vehicle control or oral doxycycline were twice that of Atridox-treated mice (213–244 mg versus 99–121 mg), which had spleens similar to normal, uninfected C3H/HeJ mice. This change in size was due to marked and expansive lymphoid hyperplasia of the white pulp with an influx of inflammatory cells, correlating with our ability to culture *A. phagocytophilum* on HL-60 cells.

It has been known for some time that the primary tick and vertebrate reservoirs for Lyme disease in the north-east and

Midwest regions of the USA are *I. scapularis* ticks and the white-footed mouse, *P. leucopus* (Bakken & Dumler, 2000; McQuiston *et al.*, 1999; Stafford *et al.*, 1999). Other pathogens, including *Babesia microti* and *A. phagocytophilum*, have been identified in these natural reservoirs, and co-transmission of these agents with *B. burgdorferi* has been well documented (Adelson *et al.*, 2004; Stafford *et al.*, 1999). It has also been reported that the duration of symptoms reported by patients co-infected with these agents exceeds that for patients with *B. burgdorferi* infection alone, making early diagnosis and successful treatment of co-infections with appropriate antimicrobial therapy imperative (Duffy *et al.*, 1997; Krause *et al.*, 2002). Our studies indicate that a sustained-release formulation of doxycycline can prophylactically block co-transmission of *B. burgdorferi* and *A. phagocytophilum* by *I. scapularis* ticks, as indicated by PCR, culture isolation and histopathology. It remains to be tested whether similar treatment with a single injection of Atridox would be more efficacious in the elimination of an early, established co-infection than the current oral dosing schedule of doxycycline in humans. Moreover, preliminary work in mice suggests that a strategy could be devised to potentially eliminate co-infection of *B. burgdorferi* and *A. phagocytophilum* in rodent and tick reservoirs in nature with other novel formulations and delivery vehicles of doxycycline hyclate. Our laboratory is currently evaluating such strategies for use in field trials to prevent the enzootic transmission of these pathogens.

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