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***Litomosoides sigmodontis*: A simple method to infect mice with L3 larvae obtained from the pleural space of recently infected jirds (*Meriones unguiculatus*)**

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Abstract

Litomosoides sigmodontis is a filarial nematode that is used as a mouse model for human filarial infections. The life cycle of *L. sigmodontis* comprises rodents as definitive hosts and tropical rat mites as alternate hosts. Here we describe a method of infecting mice with third stage larvae (L3) extracted from the pleural space of recently infected jirds (*Meriones unguiculatus*). This method enables infection of mice with a known number of L3 larvae without the time consuming dissection of L3 larvae from mites and results in higher worm recovery and patency rates than conventional methods. Additionally, this method allows for geographical separation of the facility maintaining the *L. sigmodontis* life cycle from the institution at which mice are infected.

Keywords

Litomosoides sigmodontis; filariae; nematode; helminth; *Ornithonyssus bacoti*; tropical rat mite

Introduction

The rodent filaria *Litomosoides sigmodontis* has emerged as an excellent model for studying human filariasis (Allen et al., 2008). Unlike murine infections with human filariae such as *Brugia* or *Onchocerca* species, infection of fully immunocompetent BALB/c mice with infective third stage larvae (L3) of *L. sigmodontis* results in patent infections with circulating microfilariae (Hoffmann et al., 2000; Le Goff et al., 2000). The chronic nature of *L. sigmodontis* infection in mice makes it ideal for studying immune responses to long-lived helminth infections. Indeed, the murine immune response to *L. sigmodontis* closely mimics that observed in filaria-infected humans with a well characterized development of type 2 skewed immune responses (Babayan et al., 2003; Hoffmann et al., 2000) and the generation of an immunotolerant state that occurs later in the course of infection associated with T-

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regulatory cells and the production of interleukin-10 (IL-10) (Hoerauf et al., 2005; Maizels et al., 2004; Taylor et al., 2005).

L. sigmodontis also has significant technical advantages over other murine filaria models. Unlike other models, recovery of *L. sigmodontis* worms is fairly simple and reliably complete, with over 95% of adult worms developing in the pleural cavity and easily procured by pleural lavage (Taubert and Zahner, 2001). Additionally, because the microfilariae of *L. sigmodontis* circulate in the blood, evaluation of patency can be easily done by microscopic examination of peripheral blood.

A significant challenge of working with the *L. sigmodontis* model is maintaining its complex life cycle (Fig. 1). In order to work with this helminth, a laboratory usually needs to maintain an infected colony of cotton rats (*Sigmodon hispidus*) or jirds (*Meriones unguiculatus*), a tropical rat mite (*Ornithonyssus bacoti*) colony, and laboratory mice for experimental procedures. Housing the mites is particularly challenging, requiring high humidity, 28°C room temperature, and arrangements to ensure that mites are not transmitted to other animals or humans.

Currently, laboratories that work with *L. sigmodontis* typically infect mice by direct subcutaneous injection of known numbers of L3 larvae recovered from dissection of infected mites that have fed on microfilaremic rodents (Babayan et al., 2006; Taylor et al., 2005) or unknown numbers of L3s via natural infection of mice by the vector (Al-Qaoud et al., 1997; Volkmann et al., 2003). Although these methods are effective, dissection of individual mites is highly labor intensive and natural infection of mice by the vector results in varying infection rates. In 1976, McCall demonstrated that infectious stage L3 larvae can be recovered by soaking pelts of jirds 6 hours after jird exposure to mites harboring infectious larvae (McCall, 1976). Although the pelt method is labor intensive and results in low L3 larvae recovery, it demonstrates that mite dissection can be bypassed by obtaining L3 larvae from recently-infected jirds. Petranyi & Mieth showed that L3 larvae isolated from the pleural cavity of *Mastomys natalensis* infected 7 days previously by mite exposure will develop into adult worms when injected into the pleural cavity of naïve *Mastomys natalensis* animals (Petranyi and Mieth, 1972). Taubert & Zahner adjusted this protocol by subcutaneously injecting mice with L3 larvae obtained from the pleural cavity of *M. coucha* that had been infected by mite exposure 100 hours previously (Taubert and Zahner, 2001). These pleura-derived L3 larvae successfully migrated to the pleural space of their second mammalian host and developed into adult worms which produced circulating microfilariae (Taubert and Zahner, 2001).

In this study, we tested the Taubert & Zahner protocol by investigating whether a successful murine infection could be achieved by inoculation of mice with L3 larvae obtained from the pleural cavity of naturally infected jirds at different time-points after infection.

Material & Methods

Female BALB/c mice (NCI Mouse Repository, Frederick, MD, USA) were maintained at the Uniformed Services University (USU, Bethesda, MD, USA) animal facility and jirds (*M. unguiculatus*, Charles River Laboratories, Wilmington, MA, USA) were maintained at the animal facility of TRS Laboratory Inc. (Athens, GA, USA). All animals were provided free access to food and water and all experiments were performed under protocols approved by the Institutional Animal Care and Use Committees at USU and TRS Laboratory.

The life cycle of *L. sigmodontis* was maintained at TRS Laboratory with jirds (*M. unguiculatus*) as definitive hosts and *O. bacoti* as intermediate hosts. Infected and uninfected *O. bacoti* mite colonies were maintained in stainless steel tanks equipped with electrically heated rims and boundary moats to prevent mites from escaping. The tanks were kept in an

insectary maintained at approximately 27°C and 80% relative humidity. Mites were given access to 2-4 non-anaesthetized jirds for 6-8h once a week. Mites were kept starved a week before infection. In 7 to 14 day intervals, mites from one out of three tanks were infected with *L. sigmodontis* by feeding on jirds with microfilaremias ranging from approximately 2500-10000 microfilariae per ml of peripheral blood. The infected mites were fed one more time on uninfected jirds at day 7 post infection, as an additional blood meal increases the larval number obtained from each mite (Diagne et al., 1990). Uninfected jirds in groups of three were infected at TRS Laboratory by housing them overnight in steel cages (30 cm × 38 cm × 30 cm) containing colonies of mites that had been infected 10 days previously and thus harbored infectious *L. sigmodontis* L3 larvae. While mite numbers in the tanks were hard to enumerate with precision, we estimate that jirds were usually bitten by approximately 25-200 mites. While most jirds appeared to tolerate mite feedings well, in a few cases when mite counts were high jirds became lethargic.

One day post-infection jirds were placed in a cage with an open wire floor suspended over a water-filled tub for 24 h to allow any remaining mites on the jirds to drop into the water once they had finished feeding. Three days after infection the infected jirds were transferred to an enclosed animal shipment container and shipped by overnight delivery to USU. Upon arrival to USU (day 4-6 p.i.) jirds were euthanized and pleural lavage immediately performed for the recovery of L3 larvae. Of note, careful examination of received jirds, as well as submersion of culled jirds into water, revealed no mites remaining on the shipped jirds.

Pleural lavage was conducted by careful dissection of the subdiaphragmatic space to reveal the diaphragm, creation of a 3 mm diameter hole in the ventral aspect of the right side of the diaphragm, and washing of the pleural space with a total of 15 ml of RPMI media (RPMI-1640, Mediatech, Herndon, VA, USA). Individual jird-derived L3 larvae were then aspirated into the tip of a 20 µl pipettor through use of a dissection microscope and then transferred into syringes in groups of 40.

For subcutaneous inoculation of 4- to 6-week old BALB/c mice, 40 jird-derived L3 larvae in 100-150 µl of RPMI media were injected in the dorsal neck region using a 22 gauge needle. At study endpoints, mice were euthanized and adult worms recovered and enumerated by careful dissection of the pleural cavity and examination of the peritoneum. The pleural cavity was opened with a scissor along the costodiaphragmatic recess and the worms were isolated by twirling them around a curved dissection probe. A short incubation in RPMI media at 37°C for 5-10 min increased the motility of the isolated worms and facilitated the manual separation of individual worms from worm clusters for an accurate worm count. For microfilarial counts 30 µl of blood were taken by orbital or terminal bleeding and mixed with 1 ml of ammonium-chloride-potassium (ACK) lysing buffer (Invitrogen Inc., Carlsbad, CA, USA) for 10 minutes, pelleted, and then microscopically assessed for microfilaria counts.

Statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences were tested for significance using the Kruskal-Wallis test, followed by Dunn's post-hoc multiple comparisons. P-values <0.05 were considered significant.

Results and Discussion

Prior studies have shown that *L. sigmodontis* L3 larvae migrate to the pleural cavity of rodents within 4 days (Babayan et al., 2003) and begin molting to the L4 stage approximately 8 days after infection (Bain et al., 1994; Scott et al., 1951). Therefore, L3 larvae were obtained from the pleural cavity of jirds four to six days post infection and examined for their potential to establish a new infection in susceptible BALB/c mice. Individual jirds typically harbored

several hundred L3 larvae, though the range varied (from 50 to 1000 L3 larvae) depending on the size of the mite colony and the percentages of mites harboring L3s. Jirds that were dissected earlier than 4 days p.i. had very low yields of L3s (data not shown).

Our studies demonstrated that L3 larvae recovered from the pleural cavities of recently infected jirds and injected into BALB/c mice migrated to the pleural space and established patent infections. L3 larvae obtained from 4-, 5-, and 6-day infected jirds all had the ability to establish chronic infections in BALB/c mice as adult worms were found in the pleural cavities in all challenged mice (Fig. 2). However, infection with 40 4-day-old jird-derived L3 larvae resulted in a significantly greater worm recovery rate six weeks post infection in BALB/c mice (average worm recovery 57%, median 20 worms/mouse, n=9) compared to 5-day or 6-day old jird-derived L3 larvae (25%, 9 worms/mouse, n=8, and 23%, 8 worms/mouse, n=10, respectively, Fig. 2). These results suggest that the ability of L3 larvae to establish infection decreases as the L3 larvae age. Infection with 40 4-day-old jird-derived L3 larvae resulted in patent infections in all animals studied (n=14) with an average worm recovery of 57% at 6 weeks post infection (see above) and 48% (median 14 worms/mouse, n=7) at 8 weeks post infection (Fig. 3A). The number of worms declined slightly (but not to a statistically significant degree, $p>0.05$) over time to an average worm recovery of 40% (a median of 17 worms per mouse, n=12) at 10 weeks post infection (Fig. 3A). Microfilaria counts at eight and ten weeks after infection showed median numbers of 3000 (range 1800 – 28000, n=7) and 8000 (range 1600 – 45000, n=7) microfilariae per ml of blood, respectively (Fig. 3B). As expected, no microfilariae were detected at time-points of 6 weeks p.i. (Fig. 3B) and earlier (data not shown). These results are consistent with those of Taubert & Zahner, who reported 58% adult worm recovery from mice infected 6 weeks previously with 80 L3 larvae obtained from pleural lavage of recently infected *Mastomys coucha* (Taubert and Zahner, 2001). Importantly, the percentages of jird-derived larvae which developed into adult worms and which led to circulating microfilariae in our study are higher than those reported in the literature in studies utilizing L3s obtained from mite dissection. After infection with 25 L3s obtained by dissection of infected mites, Hoffmann et al. and Petit et al. observed worm recovery in BALB/c mice of 30-40% and microfilaria counts higher than 2000 per ml in 40-60% of mice (Hoffmann et al., 2000; Petit et al., 1992). Similarly, Maréchal et al. reported worm recovery of 34% at 8 weeks post infection and 19% 10 weeks post infection and microfilaremia prevalence of 51% (Marechal et al., 1996). The higher infection rates our group and Taubert & Zahner (Taubert and Zahner, 2001) observed may be due to the fact that the L3s utilized for injection into BALB/c mice had already successfully migrated into the pleural cavity of jirds or *Mastomys* and thus this method may select for larvae with the greatest fitness to migrate to the pleural cavity. On the contrary, isolation of L3 larvae by crushing of mites might damage or reveal generally unhealthy L3 larvae which may contribute to lower infection rates. Another explanation for the greater worm recovery observed using this infection technique may be the different site utilized for L3 injection. Whereas Taubert & Zahner (Taubert and Zahner, 2001) and our group injected mice in the dorsal neck region, other investigators have used the lumbar (Marechal et al., 1996; Petit et al., 1992) or upper back area (Taylor et al., 2005) for infection, so that the L3s had to migrate further to the pleural cavity (Wenk, 1967).

In addition to finding adult worms in all mice infected with jird-derived L3 larvae, we also observed that infections with jird-derived L3 larvae resulted in detectable microfilaremia in all mice evaluated. This result is consistent with the findings of Taubert & Zahner who observed microfilaraemia in all studied mice whether they infected with 80 or 160 L3 larvae derived from *M. coucha* (Taubert and Zahner, 2001). While the exact conditions required for microfilaremia remain unknown, experiments from Babayan et al. demonstrate that infectious doses of 25 L3 larvae result in greater frequencies of microfilaremia and lesser type 2 immune responses than higher doses of 200 L3 larvae (Babayan et al., 2005). Thus, the routinely patent infections observed in our experiments may be due to the number of L3 larvae we used, the

relatively high numbers of adult worms that developed, or perhaps to selection for L3 larvae with high fitness for migrating to the pleural cavity.

In summary, recovery of L3 larvae from the pleural cavity of recently infected jirds allows *L. sigmodontis* infection of mice with a controlled number of worms without the time-consuming dissection of L3s from mites. This method results in consistently patent infections and in higher worm recovery rates than the rates reported when utilizing L3s obtained from dissection of mites. Finally, as was done in this study, this method enables the geographic separation of the facility at which the *L. sigmodontis* life cycle is maintained from the facility at which L3 larvae are procured and injected into mice.

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Figure 1.

Laboratory life cycle of *Litomosoides sigmodontis*: Generally, *L. sigmodontis* is maintained in either its natural host, the cotton rat *Sigmodon hispidus*, or in jirds, *Meriones unguiculatus*, as surrogate hosts. Infection is initiated by introduction of infective-stage L3s into the mammalian hosts by either direct injection or through the bite of infected tropical mites (*Ornithonyssus bacoti*), the natural vector. After inoculation, larvae migrate via the lymphatic system to the pleural cavity by d2-6 post-infection (p.i.), where subsequent development takes place. Larvae molt to L4 stage larvae between d8-12 p.i., become adults by d25-30p.i., and are able to produce microfilariae which circulate in the blood by d50p.i.

Fig. 2. Number of worms recovered 6 weeks after infection of BALB/c mice with 40 L3 larvae obtained from the pleural cavity from 4-day, 5-day, or 6-day infected jirds. Significant differences between groups were analyzed by the Kruskal-Wallis test, followed by Dunn's post-hoc multiple comparisons (* $p < 0.05$).

Fig. 3.
A) Number of worms and B) microfilariae recovered 6, 8 or 10 weeks after infection of BALB/c mice with 40 L3 larvae obtained from the pleural cavity from 4-day infected jirds.