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Trichinella spiralis: Differential effect of host bile on the *in vitro* invasion of infective larvae into epithelial cells

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ABSTRACT

The differential effect of fox and pig bile and its corresponding low molecular weight fraction (LMW) was investigated on the *in vitro* invasion of MDCK-AA7 epithelial cell monolayers by *Trichinella spiralis* muscle larvae. Seven invasion experiments were performed and a total of 274 cell monolayers were examined. Fox and pig raw bile at 1:10 and 1:20 dilution and their LMW fractions at 1:10 dilution activated *T. spiralis* larvae to invade the cell monolayers. In addition, fox raw bile caused significantly larger cell damage than pig raw bile at both dilutions. The area of cell damage was larger at 1:10 than at 1:20 dilution for both fox and pig raw bile ($p < 0.05$). On the other hand, there was no significant difference between the areas of cell damage caused by the LMW fractions of fox and pig bile. It is concluded that differences between host bile actions may account for differences in host susceptibility to *T. spiralis*.

1. Introduction

Members of the genus *Trichinella* are parasitic nematodes that infect their vertebrate host after consumption of meat containing infective larvae. Following their release in the stomach by digestion of the meat, the larvae penetrate into host's intestinal epithelium where they molt and develop to adults that mate and reproduce the next generation of larvae. Adults and newborn larvae colonize several epithelial cells simultaneously migrating in a serpentine pattern leaving behind a trail of dead cells (Dunn and Wright, 1987; Wright, 1979), but do not invade other cell types, such as fibroblast or muscle cells (ManWarren et al., 1997). The mechanism of epithelial cellular invasion by the larvae is not known, in fact the mere delivery of glycoproteins or mechanical wounding is insufficient to allow entry of the parasite into resistant epithelial cells (Butcher et al., 2000). The life cycle of the parasite is concluded after the newborn larvae migrate through the circulatory system and establish into striated muscle cells where they mature to infective larvae awaiting to pass onto the next host (Despommier, 1983).

Most *Trichinella* species are able to complete their entire life cycle in many hosts, but as a result of host adaptations, some species have limited infectivity. Foxes have a high susceptibility to all species of *Trichinella* spp., while rodents and herbivorous animals have a significantly higher susceptibility to domestic *Trichinella spiralis* than sylvatic species (Kapel, 2000). The mechanism underlying these observations is not known, but previous studies have shown that diluted raw fox bile improved the establishment of *T. nativa* and *T. nelsoni*, but not *T. spiralis* in rats (Theodoropoulos et al., 2003) and that the non-protein fraction of bile at various dilutions prolonged the survival of *Trichinella* larvae *in vitro* (Theodoropoulos et al., 2005a), while larvae incubated in diluted bile invaded host cells *in vitro* more aggressively than larvae not exposed to bile (ManWarren et al., 1997).

The objective of the present study was to investigate the differential effect on the *in vitro* invasion of epithelial cells by *T. spiralis* muscle larvae following their activation by fox and pig bile and its corresponding low molecular weight fraction. The study was based on the invasion assay developed by ManWarren et al. (1997) who demonstrated that it reproduces the broad host range and restricted cell specificity of the parasite *in vivo*.

2. Materials and methods

2.1. Species and source of muscle larvae and bile

The *T. spiralis* (pig strain) was maintained by serial passages in adult AO strain irradiated rats produced and maintained in the

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James A. Baker Institute vivarium in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care. The larvae were released from rat muscle tissue by digestion (1 l ddH_20, 10 g 1:10.000 IU Pepsin, 10 ml 37% HCl) for 1 h at 37 °C and recovered on a 200-mesh sieve and then washed with saline. The larvae were prepared and used fresh right before experimentation.

Bile was collected from farmed foxes and pigs at slaughter and stored at −80 °C prior to experimentation. The low molecular weight fraction of bile (LMW) was obtained by filtering raw bile using a centrifugal device with a 10,000 Nominal Molecular Weight Limit (Amicon Ultra 10 K).

2.2. Cell line

The MDCK-AA7 epithelial cell line from dog kidney was used. The AA7 cells were cultured (5% CO_2, 37 °C) in 75 cm^2 culture flasks (CORNING) containing Minimal Essential Medium, with 2 mM L-glutamine (GIBCO) and 10% heat inactivated Foetal Calf Serum (Hyclone).

2.3. Activation of muscle larvae

The muscle larvae were activated as previously described by ManWarren et al. (1997). Briefly, muscle larvae were incubated (2 h, 37 °C, shaking every 15 min) with Earle's Balanced Salt Solution (EBSS) diluted raw fox bile (1:10 or 1:20), raw pig bile (1:10 or 1:20), or their LMW fraction (1:10). In addition, larvae were activated with EBSS diluted luminal content of rat intestines (1:10) that served as positive control, as well as with EBSS diluted saline (1:10) that served as negative control.

2.4. Cell monolayers

Monolayers of AA7 cells were grown to confluence on chamber eight well glass slides (Lab-Tek Chamber Slide w/cover sterile by Nalge Nunc International). The monolayers were prepared as previously described by ManWarren et al. (1997). Cell monolayers were plated the day before experimentation and were checked for confluence right before overlaying the larvae. Monolayers that were not confluent were not used.

2.5. Invasion assay

Following activation a total of 40 larvae were overlaid into each well on the AA7 cell monolayers in the chamber slide wells and incubated as previously described by ManWarren et al. (1997). After incubation the number of serpentine vs. spiral larvae in contact with the monolayers was counted by using a phase-contrast inverted microscope and a 4× objective. The serpentine and the spiral larvae were considered as activated and non-activated, respectively (Fig. 1). Then the monolayers were washed with Dulbecco's PBS plus $MgCl_2$ and $CaCl_2$ to remove the agarose and processed for microscopy. The slides were examined for trails and measuring cell damage on the monolayers caused by the larvae. Only slides with trails in the positive control monolayers and no trails in the negative control monolayers were taken into account.

2.6. Measurements

During invasion of the cell monolayers the larvae migrated in a serpentine pattern leaving behind a trail of damaged cells that stained blue with trypan (Fig. 2a). The area of damaged cells was determined with specialized software (Image Pro Plus™, Version 3.0; MEDIA CYBERNETICS®, USA) which isolated the area of interest for measurement after clearing any debris in the observed microscope field (Fig. 2b).

2.7. Statistical analysis

The data generated during the experiments were the measurement of damaged area on each cell monolayer, as well as the total number of larvae and the number of activated larvae in contact with the monolayer. Even though in principle the number of larvae overlaid on each cell monolayer to perform the invasion assay was the same in all slide wells, in practice there was a variation in their numbers due to experimental error which may have had affected the measurements of the damaged area and consequently any differences between bile treatments. In a preliminary analysis it was found that the damaged area was positively correlated (Table 1) with the number of activated larvae but not with the total number of larvae. For this reason the number of activated larvae was deemed to have a causal effect on the damaged area and was used in subsequent analyses.

In order to eliminate the possible effect of the unequal number of activated larvae on the measurements of the damaged area, the damaged area data was transformed by calculating the ratio of the measurement of damaged area divided by the number of activated larvae in contact with the monolayer. The effect of the number of activated larvae on the damaged area as well as on the ratio of the damaged area by the number of activated larvae was assessed by calculating the Pearson correlation. Normality of data was assessed by using the Colmogorov–Smirnov goodness of fit normality test.

The effect of bile treatments on the measurement of damaged area was analyzed using the following one way ANOVA model:

$$\mathrm{Ln}\,(Y_{ij}/AL_{ij}) = \mu_i + \varepsilon_{ij}$$

whereas, Y_{ij} is the measurement of damaged area in the jth trial for the ith factor (treatment), AL_{ij} = number of activated larvae in the jth trial for the ith factor (treatment), μ_i are parameters (mean damaged area for i treatment level), ε_{ij} = are independent normal random errors i.i.d. $N(0, \sigma^2)$, $i = 1,2\ldots,r$ (r = total number of treatment), $j = 1,2,\ldots n_i$.

A p value of <0.05 indicates a significant difference in response variable among different treatments ($\mu_i \neq \mu_{i'}$, i, $i' \in \{1,2,\ldots r\}$).

3. Results

A total of seven invasion experiments were performed and a total of 274 cell monolayers were examined. The Pearson correlation between the damaged area and the number of activated larvae was found small but significant ($p < 0.05$). This correlation became insignificant when the damage area variable was replaced by its ratio with the number of activated larvae (Table 1). Therefore this ratio was used as dependent variable in order to detect differences in damaged area measurements between bile treatments.

The Colmogorov– Smirnov goodness of fit normality test indicated a serious departure from normality of the dependent variable (p-value < 0.01). For this reason the natural log (ln) transformation of these data was used. Because the negative control values were zero and ln (0) cannot be determined within the set of real numbers, all negative controls were not taken into account in the statistical analysis and results presented in the tables.

Fox and pig raw bile and their LMW fractions activated *T. spiralis* larvae to invade the cell monolayers. In addition, fox raw bile caused significantly larger cell damage than pig raw bile at both 1:10 and 1:20 dilution. The area of cell damage was larger at 1:10 than at 1:20 dilution for both fox and pig raw bile ($p < 0.05$) (Table 2). On the other hand, there was no significant difference between the areas of cell damage caused by the LMW fractions of fox and pig bile.