Studies on the viability of metacercariae of *Fasciola gigantica*

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Abstract

The viability of metacercariae of *Fasciola gigantica* was tested by *in vitro* and *in vivo* methods. *In vitro* testing was based upon the motility of juvenile flukes within the inner cyst as examined under the light microscope. *In vivo* testing was undertaken through experimental infections of rabbits (two groups) and natural definitive hosts, lambs (one group). In the first group, out of six rabbits each given 25 metacercariae, worm establishment only took place in one rabbit with a single fluke recovery on 60 days post infection. In the second group of six rabbits each given 200 metacercariae, five were infected, with two or three flukes per host. All the lambs given 250 metacercariae became infected showing prevalences of 7.2–40% in comparison with rabbits in which low prevalences (0–4%) were recorded. The results indicated that even viable metacercariae which were already tested *in vitro* could not readily establish in rabbits. Such variability in worm establishment suggests that immunological and chemotherapeutic studies in rabbits infected with *F. gigantica* are likely to be unreliable.

Introduction

Metacercariae are the infective stages of *Fasciola gigantica* used in experimental infections in laboratory mammals as well as in natural definitive hosts. During our preliminary studies, rabbits did not show worm establishment in many cases in contrast to lambs, in which infections were successfully established. Several reports describe the failure of development of *F. gigantica* in rabbits routinely used as laboratory hosts (Thapar & Tandon, 1952; Srivastava & Singh, 1974; Graber, 1974; El-Bahy, 1997). The majority of workers used metacercariae of variable age without testing the viability *in vitro* before administrating to the experimental animals in the laboratory (Thapar & Tandon, 1952; Mango *et al*., 1972; Khajuria & Bali, 1987). Moreover, in most cases the strain of rabbit is not mentioned. Age and viability of metacercariae were also unknown.

Therefore, the aim of the present study was to determine whether the establishment of *F. gigantica* was due to host species (using rabbit and lambs) or metacercarial viability and also whether or not the rabbit should be used as a laboratory model for chemotherapeutic and immunological studies on *F. gigantica*.

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Materials and methods

Collection of metacercariae

Cercariae of *F. gigantica* were obtained from experimentally infected *Lymnaea auricularia rufescens*. Snails were maintained at pH 7.6–8.4 and a temperature of 25–27°C in water. The snails were provided with an artificial diet as described by Malek & Cheng (1974) but with slight modifications (Prasad, 1989). The artificial diet consisted of 10 g dry spinach leaf powder, 2.5 g baby cereal food (Glaxo Lab.), 5 g powdered wheat germ and 3 g calcium carbonate. To avoid contamination of the water, an optimum amount of feed was provided. Metacercariae were collected on transparent polyethylene sheets in the morning as cercariae emerge from the snails overnight. Metacercariae were stored in sterile conditions at 4°C.

In vitro *experiments*

A batch of 25 metacercariae, 30 days old of buffalo origin, were used for *in vitro* experiments. The outer cyst was removed by compressing with a needle so that the inner cyst emerged. Viability was determined as previously described by Hanna *et al*. (1975). Only those metacercariae which showed movement within the cyst

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and also possessed clear excretory granules, oral and ventral suckers were considered suitable for *in vivo* experiments.

In vivo *experiments*

Experimental animals

Twenty four New Zealand white rabbits, 1–2 months old, were housed individually in cages and fed on standard rabbit cubes and water *ad libitum*. Rabbits were divided into four groups (I, II, III, IV) with six animals in each group.

Eight clean healthy lambs, 4–5 months old, were given food and water *ad libitum* and divided into two groups (V and VI) with four animals in each group. Enzyme linked immunosorbent assays (ELISA) were performed using somatic antigen of *F. gigantica* prepared as previously described by Zimmerman *et al*. (1982).

Experimental design

Experimental animals were treated as follows: groups I, II and V were infected orally with 25, 200 and 250 viable metacercariae respectively. Groups III, IV and VI were kept as controls for group I, II and V respectively. Animals were inoculated with viable metacercariae in water. Inoculation doses were prepared after counting the metacercariae under the microscope and specific numbers of metacercariae were then pipetted into 5 ml glass tubes. During inoculation care was taken to ensure that all the metacercariae were ingested by the experimental animals.

Sera

Blood was collected weekly from the animals of group V and VI commencing 2 weeks prior to infection up to 60 days post infection (DPI).

Enzyme-linked immunosorbent assay

Antigen

Adult *F. gigantica* were collected in chilled normal saline solution (NSS) from the liver of sheep, thoroughly washed with NSS followed by cold 0.01 M phosphate buffered saline (PBS) pH 7.2. Flukes were then homogenized in 0.01 M PBS containing phenylmethane sulphonyl fluoride. The homogenate was sonicated at 8 μm for 10 min interspersed with periods of cooling. The sonicated suspension was centrifuged at 12000 *g* for 45 min at 4°C. The supernatant was collected, filtered through 0.45 μm filter (Sartorious Biotech, India) and the filtrate was used as somatic antigen (SAg).

Test procedure

Microtitre plates (Titertek) were coated with 100 μl of SAg (20 μg ml^{-1}) in carbonate bicarbonate buffer, pH 9.6. After overnight incubation at 4°C (Harlow & Lane, 1988) and washing the plates 4 times with PBS containing 0.05% Tween-20 (PBST), the plates were blocked with 300 μl of PBS containing 1% BSA per well for 2 h at room temperature. After washing the plates with 4 changes of PBST, 100 μl of 1:100 dilution of experimental and control sera collected on different days in 1 × PBS were placed in triplicate wells and kept at room temperature for 2 h. The plates were again washed with PBST for 4 times, 100 μl of a 1:1000 dilution in 1 × PBS of affinity purified donkey antisheep IgG (Sigma, USA) peroxidase conjugate were added to each well. After 2 h incubation, the plates were washed and finally 100 μl of substrate solution containing O-phenylene diamine (Sigma, USA) in phosphate citrate buffer, pH 5.0 and H_2O_2 were added to each well. The plates were kept in darkness for 30 min and optical density was measured at 492 mm using a Titertek multiscan plate reader (Flow Labs, UK).

Fluke recovery

Worms were recovered at 60 DPI. Immature flukes were recovered from the liver tissue using pepsin digestion after chopping the liver. Counts were made of the total number of flukes recovered and the worm sizes recorded.

Results

Movement of juvenile flukes occurred in 100% of cases within the inner cyst of metacercariae when test batches were examined.

Before infection, the antibody response was not detected in any of the test animals. However, following ingestion of metacercariae, a significant ($P < 0.01$) antibody response was recorded at 35 DPI with the highest response being recorded at 56 DPI compared with controls (fig. 1).

The mean number of flukes recovered from groups I, II, III and IV are shown in table 1. In group I, only one fluke

Table 1. Viability of metacercariae of *Fasciola gigantica* and worm establishment in rabbits and lambs at 60 days post infection.

Host group	No. of hosts in each group	No. of Mc given *per os* per host	*In vitro* viability of Mc (%)	No. of hosts infected	No. of worms recovered	Worm establishment (%)	Host susceptibility (%)
Rabbit							
Group I	6	25	100	1	0, 0, 0, 0, 0, 1	0–4	16.7
Group II	6	200	100	5	0, 2, 2, 3, 3, 3	0–1.5	83.3
Lamb							
Group V	4	250	100	4	18, 35, 56, 100	7.2–40	100.0

Rabbit groups III and IV and lamb group VI were uninfected.

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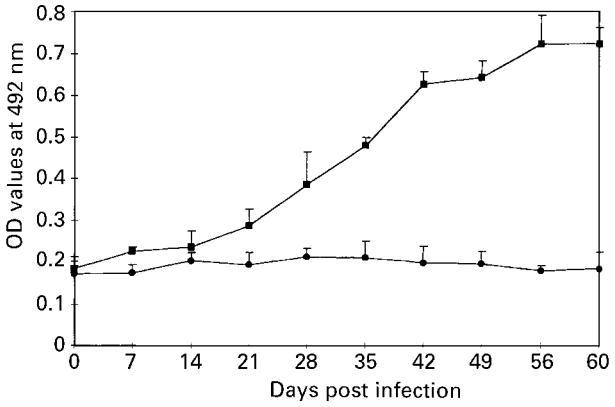

Fig. 1. Antibody response to *Fasciola gigantica* in experimentally infected (■) and control (●) lambs up to 60 days post infection.

was recorded, whereas in group II, worm establishment occurred in only 1.5% of cases. No flukes were recovered from rabbits in groups III and IV.

All lambs showed a 100% infection whereas worm establishment was significantly ($P < 0.01$) higher in lambs when compared with rabbit (table 1) and worms were also larger in lambs. Worm size in lambs was 10.98–11.65 × 2.28–2.4 mm compared with 3.96–4.0 × 1.8–2.0 mm in rabbits.

Discussion

Testing the viability of metacercariae of *F. gigantica* through *in vitro* methods indicates that 30-day-old metacercariae stored at 4°C possess clear internal structures and are 100% viable (Hanna *et al*., 1975). The viability of metacercariae when re-tested using immunological methods shows significant antibody responses in lambs at 35 DPI reaching a maximum at 56 DPI. Immune responses following infection with metacercariae were also reported by Poitou *et al*. (1992) and Guobadia & Fagbemi (1995) in rats and sheep respectively.

Worm establishment is regarded as one of the criteria for determining infectivity of parasites and in the case of *F. gigantica* it was surprising to note that only one worm was recovered from one rabbit in group I whereas in group II rabbits, worm establishment ranged between 0 and 3. On the other hand, significant worm establishment was recorded in lambs of group V, which further established the viability of the metacercariae. These results indicate that variation in worm establishment may be due to individual susceptibility of rabbits and not necessarily due to the viability of metacercariae, since metacercariae of similar ages were used during the experiment and viability was also tested *in vitro*. Our results are supported by several workers both in terms of viability of metacercariae as well as worm establishment. Srivastava & Singh (1974) obtained variable results during experimental infections of rabbits with different doses of metacercariae of varying age. An infective dose of 1000 metacercariae (12 days old) established the infection with four flukes only, but a similar dose of 18- and 30-day-old metacercariae did not establish. This indicates that the metacercarial age and the infective dose are not necessarily related to worm establishment. This is supported by our results in which an infective dose of 200 metacercariae (30 days old) established an infection in rabbits. Thapar & Tandon (1952) were unable to establish infections in rabbits given 256 metacercariae (age unknown). Mango *et al*. (1972) established infections in 61% and 20% of experimental rabbits with oral doses of 25 and 5 metacercariae (age unknown) respectively. However, Kimura & Shimizu (1978) obtained a 60% worm establishment in one rabbit and 50% in another rabbit with an infective dose of 20 metacercariae (3 days old). Yoshihara & Goto (1993) recorded 5, 8 and 4 adult worms with an infective dose of 50 metacercariae administered to specific pathogen free male rabbits. These results not only indicate that worm establishment varies considerably in the same group of rabbits but also support our views that it is related to individual susceptibility in rabbit which is not a natural host for *F. gigantica*.

Recently, Yoshihara *et al*. (1995) suggested that the separation of the outer cyst of the metacercariae and inoculation of separated metacercariae results in more successful worm establishment when compared with conventional methods of inoculation. These workers used mice as the laboratory model and variability in worm establishment was recorded. Two groups of mice were fed with metacercariae of *F. gigantica* using two different methods. Using the agar gel method, worm establishment in one group ranged between 0 and 4, whereas in the other group it was 2 and 7. In the agar gel method, metacercariae with outer cysts were placed on 5% agar gel in a tube, inoculated orally by inserting a tube, attached to a syringe, into the oesophagus of each mouse. Conversely, Yosihara *et al*. (1995) inoculated separated metacercariae orally with the help of a syringe but without using the agar gel method. Metacercariae were separated by an aqueous two phase system, originally developed to clean bacterial spores, using polyethylene glycol and dextran. Inoculation of the metacercariae resulted in a higher worm establishment in mice compared with the group receiving metacercariae through the agar gel method. They were of the opinion that the loss of metacercariae during inoculation in the agar gel method resulted in a lower worm establishment. But in our opinion worm establishment is also related to host susceptibility and viability of the metacercariae.

Moreover, Yoshihara *et al*. (1995) did not refer to the age of the metacercariae and this is an important criterion for determining viability. They found 15% of the metacercriae non-viable, suggesting that either the metacercariae were already non-viable or they had lost their viability due to toxicity of the chemicals used in the separation method.

In the present study, it has been shown that viability of metacercariae cannot be determined through the *in vivo* method as infections did not always materialize. These studies are further supported by Hanna *et al*. (1975) who indicated that hatching of the juvenile flukes from metacercariae through excystment does not necessarily result in invasion of the host intestine nor produce infections in the liver. Furthermore, the present study has shown that metacercariae of similar ages can establish infections of *F. gigantica* in lambs which are the natural

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definitive hosts but not in rabbits which are natural reservoir hosts for *F. hepatica*. On the other hand, Yoshihara & Goto (1993) obtained adult *F. gigantica* from the liver of three rabbits, suggesting that either the Japanese liver fluke is a different strain or the rabbits used in their experiments were a more susceptible strain. The existence of a Japanese strain of *F. gigantica* receives further support as Yoshihara & Goto (1993) recovered adult worms in mice, although in India the strain of *F. gigantica* does not develop in mice (Srivastava & Singh, 1974; Khajuria & Bali, 1987). It is, therefore, reasonable to suggest that differences in the susceptibility and development of *F. gigantica* in the same laboratory animals implies that geographical and physiological differences operate between the strains of *F. gigantica* (Coyle, 1959; Mango *et al*., 1972). As reported earlier by El-Bahy (1997) the present observation reaffirms that the routinely used rabbit strain (New Zealand white) in India is not a suitable laboratory model.

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