

A method for the isolation of schistosome eggs and miracidia free of contaminating host tissues

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SUMMARY

A novel method for the isolation of schistosome eggs and miracidia from livers of mice infected with *Schistosoma japonicum* or *S. mansoni* is described. The method employed collagenase B to degrade the interstitial matrix of mouse liver tissue, after which the schistosome eggs were separated from the liver cells by 2 single-step density centrifugations through Percoll. Using this procedure sufficient quantities of miracidia were obtained to generate a cDNA library. Southern blot analysis demonstrated that miracidia isolated by this method were free from contaminating host DNA.

Key words: *Schistosoma mansoni*, *Schistosoma japonicum*, eggs, miracidia.

INTRODUCTION

The pathology associated with chronic schistosomiasis *mansoni* and schistosomiasis *japonica* primarily results from granulomatous responses to eggs entrapped within the tissues and organs, particularly the liver and intestines, of the host (see Mitchell *et al.* 1994). Although the mediators that induce these T cell-dependent responses have not been defined, these are likely to be soluble egg antigens (Boros & Warren, 1970; Colley, 1981; Wynn *et al.* 1995). Recent reports have also implicated proteolytic enzymes, such as cathepsin B and cathepsin L, released from schistosome eggs as being responsible for host liver pathology (Asch & Dresden, 1979; Pino-Heiss, Brown & McKerrow, 1985; McKerrow & Doenhoff, 1988; Day *et al.* 1995).

Because of increasing interest in proteolytic enzymes of eggs and miracidia (Day *et al.* 1995), and the cDNAs encoding these proteases, there is now a requirement to isolate larval stages of schistosomes free of contaminating host tissues. The method described by Baltz *et al.* (1982) for the isolation of eggs from hamster liver employed both filtration and density-gradient centrifugation, and was an improvement from earlier procedures that involved tissue degradation with endogenous proteases or exogenous trypsin (Smithers, 1960; Browne & Thomas, 1963; Asch & Dresden, 1979; Pino-Heiss *et al.* 1985), sieving (Dresden & Payne, 1981) and a combination of filtration and sedimentation (Coker

& Von Lichtenberg, 1956; Ritchie & Berios-Duran, 1961; James & Colley, 1974). However, in our laboratories we found that eggs isolated using the method of Baltz *et al.* (1982) remained contaminated with host tissue debris. While contamination with host tissues might be inconsequential for some applications, for instance for maintenance of the life-cycles, it can be problematic if the eggs and/or miracidia are to be used as a source of nucleic acids for construction of gene libraries or other molecular biology techniques. Indeed, the contamination of schistosome nucleic acids with mammalian sequences has been shown to occur frequently (Iwamura & Irie, 1992; Irie & Iwamura, 1993; Franco *et al.* 1995; Clough, Drew & Brindley, 1996), the consequences of which have led to considerable controversy in interpretation of results claiming the presence of host-like sequences in the schistosome genome (Simpson & Pena, 1991; Iwamura, Yonekawa & Irie, 1995; Clough *et al.* 1996). Therefore, we undertook the development of an improved method for egg isolation, cognisant of the apparent shortcomings of the strategies that had been previously employed. We now report a procedure that proved to be reliable for the isolation of eggs free of host cell contamination.

MATERIALS AND METHODS

Materials

Polypropylene tubes were from Disposable Products, Technology Park, South Australia. Fifty ml Falcon tubes were purchased from Becton Dickinson Labware, Lincoln Park, NJ, USA. Collagenase B was from Boehringer Mannheim, Australia. Peni-

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cillin and streptomycin were purchased from CSL, Parkville, Australia. The Qiagen genomic columns were from Qiagen, Hilden, Germany, the Hybond-N nylon from Amersham, Sydney, NSW, Australia, and Percoll from Pharmacia, Uppsala, Sweden.

Isolation of eggs from mouse livers

Three to four livers from mice infected with *S. mansoni* or *S. japonicum* were chopped finely with a scalpel blade, and then blended to a smooth consistency in 50 ml of phosphate-buffered saline (PBS). The suspension was decanted into a 50 ml Falcon tube. Twenty mg collagenase B, 100 μ l (10 μ g) of penicillin and 100 μ l (20 μ g) of streptomycin (CSL) were added to the tube, and the mixture incubated with shaking at 37 °C overnight. The tube was then centrifuged at 400 g for 5 min, the supernatant removed and the pellet resuspended in 50 ml of fresh PBS. This wash procedure was repeated twice more, with the exception that after the final centrifugation the pellet was resuspended into 25 ml of PBS. The resuspended mixture was passed through a 250 μ m sieve, and then a 150 μ m sieve. The filtrate was centrifuged at 400 g for 5 min, the supernatant discarded and the pellet resuspended in 3 ml of PBS and applied to the top of a Percoll column, prepared by mixing 8 ml of Percoll with 32 ml of 0.25 M sucrose in a 50 ml Falcon tube. The tube was centrifuged at 800 g for 10 min. Liver cells remained on the top of the Percoll and were removed with a Pasteur pipette. The schistosome eggs, which pelleted tightly at the bottom of the tube, were then removed with a Pasteur pipette. Eggs were washed 3 times with PBS containing 1 mM EDTA, 1 mM EGTA in a 10 ml tube at 30 g. The metal chelators were included to inactivate the collagenase B. Residual liver cells were removed by discarding the supernatants. Further purification of eggs was achieved by resuspension in 0.5 ml of PBS and application on to a second Percoll column, prepared by mixing 2.5 ml of Percoll with 7.5 ml of 0.25 M sucrose in a 10 ml polypropylene tube. The eggs were pelleted and then washed as before, with the exception that EDTA and EGTA were omitted from the wash buffers.

Isolation of miracidia

Eggs were added to 0.5 ml of distilled water in a microfuge tube and exposed to a bright light (Pino-Heiss *et al.* 1985). Miracidia hatched within 15 min, and the supernatant was removed every 30 min for 2 h and replaced by fresh water. The supernatants containing the miracidia were pooled, chilled on ice (miracidia lose motility in cold water and sink to the bottom) and miracidia were then recovered by centrifugation at 15 000 g for 20 sec at 4 °C using an Eppendorf microcentrifuge.

Southern hybridization

We carried out Southern hybridization experiments in order to demonstrate whether genomic DNAs from miracidia purified by this procedure were free of contaminating host DNA and, by implication therefore, free of contaminating host cells. Mixed-sex adults and miracidia of *S. mansoni* (Puerto Rican strain) were obtained from mice 7–10 weeks after an experimental infection with 100 cercariae. Genomic DNAs from these schistosomes were isolated by chromatography using Qiagen genomic columns according to the manufacturer's instructions. Rather than use the genomic DNAs in our Southern hybridizations, we employed the strategy of amplicon construction, as described by Lisitsyn, Lisitsyn & Wigler (1993), in order to enhance the sensitivity of the hybridizations (Drew & Brindley, 1995). The amplicon construction uses the polymerase chain reaction (PCR) to amplify 'representations' of genomic DNA. *Hind* III-derived amplicons were constructed from miracidial and adult schistosome DNAs as previously described (Drew & Brindley, 1995). Approximately 1 μ g of each amplicon of genomic DNA was then separated by agarose gel electrophoresis and Southern transferred to Hybond-N nylon by standard techniques. The nylon filter was then probed with a ³²P-labelled, *Hind* III-fragment of a mouse repetitive DNA (approximately 1000 copies/mouse genome encoding intra-cisternal A particle, Burt, Reith & Brammar, 1984) isolated as described by Clough *et al.* (1996). The filter was subsequently stripped of this probe by boiling in 0.1% SDS after which it was reprobed with a ³²P-labelled, *Hind* III-fragment of an *S. mansoni* repetitive sequence (GenBank accession no. U66336).

RESULTS/DISCUSSION

The procedure outlined above worked equally well for the isolation of *S. mansoni* and *S. japonicum* eggs. The yield of eggs was dependent on intensity of the schistosome infection but, as an indicator, we routinely obtained approximately 2000 eggs/mouse liver obtained from animals 7 weeks after infection with 30 *S. japonicum* cercariae. Approximately 75% of the eggs hatched and released miracidia (Fig. 1). We have constructed a cDNA library in Uni-ZAP XR (Stratagene, Integrated Sciences, Sydney, Australia) using mRNA isolated from 12 500 miracidia obtained from 14 livers of *S. japonicum*-infected mice by employing the described procedure. The library was >60% recombinant with an average insert size of 975 bp and a titre of 3.2×10^9 pfu/ml.

The mouse intra-cisternal A particle sequence hybridized to sequences of host cell origin in the amplicon prepared from adult schistosome DNA (Fig. 2, lane 1). This observation was expected since

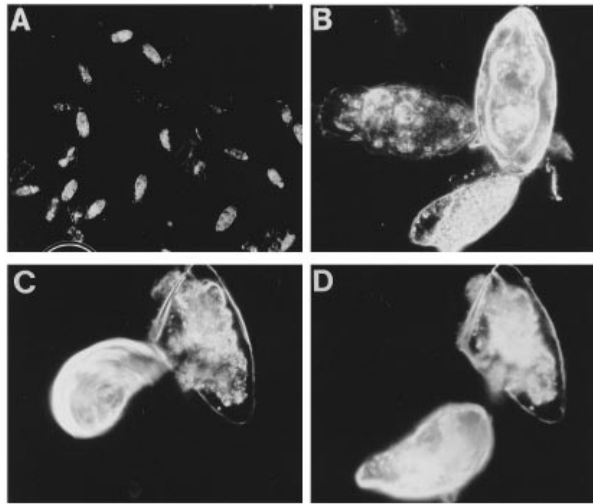


Fig. 1. Samples of purified eggs of *Schistosoma mansoni* viewed by microscopy ($\times 200$) (A). Under high power fully embryonated miracidia can be observed (B). Miracidia hatch from the eggs within 5 min of placing eggs in water (C and D).

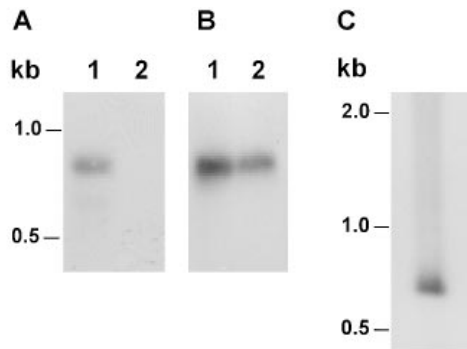


Fig. 2. Southern blotting analysis of schistosome adult and miracidial DNAs. Adult (lanes 1) and miracidial (lanes 2) *Schistosoma mansoni* *Hind* III amplicons were probed with a cloned fragment of DNA from the mouse intra-cisternal A particle (A) and a cloned *S. mansoni* repetitive DNA fragment (B). Mouse genomic DNA digested with *Hind* III and probed with the mouse intra-cisternal A particle served as a positive control (C).

adult worm DNA preparations are often contaminated with host DNA (see Clough *et al.* 1996). In contrast, this probe did not hybridize to the miracidial amplicon DNA (Fig. 2, lane 2). The *S. mansoni* repetitive sequence hybridized to both adult and miracidial amplicon DNAs at a comparable level (Fig. 2, lanes 1 and 2), which indicated that the differences in the level of host DNA hybridization were not due to differences in quantity of DNA in the separate lanes. Taken together, these data showed that miracidia purified by our method contained no detectable, contaminating host DNA and, therefore by extension, no contaminating host cells.

In summary, this paper describes a methodology for the isolation of *S. mansoni* and *S. japonicum* eggs from mouse livers. The method takes advantage of the substrate specificity of the metalloproteinase

collagenase B for the digestion of interstitial collagen, which has long been employed in methods for the isolation of hepatocytes (Tager, Rubenstein & Steiner, 1975). When livers from schistosome-infected mice are incubated overnight with this enzyme a fine suspension of eggs and cells is obtained. The eggs can then be separated from host cells by sieving followed by centrifugation through a single layer of Percoll. Sufficiently pure eggs for experiments such as those that investigate egg hatching can be obtained using a single Percoll purification step. However, eggs and miracidia which are totally free of detectable contamination with host tissues can be obtained by employing a second Percoll purification. In our laboratory these procedures provided sufficient material to allow the preparation of a miracidial cDNA library. We consider that the procedure outlined here should be of use for the identification and characterization of the schistosome egg antigens (particularly by recombinant DNA techniques), including those that are apparently responsible for the hepatic fibrosis associated with schistosomiasis *mansoni* and schistosomiasis *japonica*.

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