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An antibiotic selection marker for schistosome transgenesis

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ABSTRACT

Drug selection is widely used in transgene studies of microbial pathogens, mammalian cell and plant cell lines. Drug selection of transgenic schistosomes would be desirable to provide a means to enrich for populations of transgenic worms. We adapted murine leukaemia retrovirus vectors – widely used in human gene therapy research – to transduce schistosomes, leading to integration of transgenes into the genome of the blood fluke. A dose–response kill curve and lethal G418 (geneticin) concentrations were established: 125–1,000 μg/ml G418 were progressively more toxic for schistosomules of *Schistosoma mansoni* with toxicity increasing with antibiotic concentration and with duration of exposure. By day 6 of exposure to ⩾500 μg/ml, significantly fewer worms survived compared with non-exposed controls and by day 8, significantly fewer worms survived than controls at ⩾250 μg/ml G418. When schistosomules were transduced with murine leukaemia retrovirus encoding the neomycin resistance (*neoR*) transgene and cultured in media containing G418, the *neoR* transgene rescued transgenic schistosomules from the antibiotic; by day 4 in 1,000 μg/ml and by day 8 in 500 μg/ml G418, significantly more transgenic worms survived the toxic effects of the antibiotic. More copies of *neoR* were detected per nanogram of genomic DNA from populations of transgenic schistosomes cultured in G418 than from transgenic schistosomes cultured without G418. This trend was G418 dose-dependent, demonstrating enrichment of transgenic worms from among the schistosomules exposed to virions. Furthermore, higher expression of *neoR* was detected in transgenic schistosomes cultured in the presence of G418 than in transgenic worms cultured without antibiotic. The availability of antibiotic selection can be expected to enhance progress with functional genomics research on the helminth parasites responsible for major neglected tropical diseases.

1. Introduction

Schistosomiasis is considered the most important of the helminth diseases in terms of morbidity and mortality with more than 200 million infected people and a further 800 million at risk. Treatment and control of this neglected tropical disease relies on a single drug, praziquantel. New interventions including vaccines, drugs and diagnostics are needed as a global health priority (Hotez et al., 2008; Brindley et al., 2009). Genome sequences for *Schistosoma japonicum* and *Schistosoma mansoni* were reported recently; landmark events that ushered in the post-genomic era for schistosomiasis (Berriman et al., 2009; *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium, 2009). Analysis of target genes to underpin new interventions for schistosomiasis would be aided by functional genomics to validate the essentiality of gene functions to be targeted with novel drugs or vaccines. For *Caenorhabditis elegans*, methods for genetic manipulation are well advanced. In noteworthy recent progress on the technical toolkit for *C. elegans*, drug selection with antibiotics, neomycin and puromycin has been demonstrated utilising resistance genes perpetuated in the transgenic worms as extrachromosomal arrays (Giordano-Santini et al., 2010; Semple et al., 2010). A molecular toolkit would also enhance our capacity to perform genetic manipulations of schistosomes and other parasitic helminths and recently there has been progress in this endeavour (Grevelding, 2006; Castelletto et al., 2009; Suttiprapa et al., in press-a). For example, murine leukaemia retrovirus (MLV) vectors – widely used in human gene therapy research – have been adapted to transduce schistosomes, leading to chromosomal integration of reporter transgenes and transgene expression (Kines et al., 2008). As noted in regard to advances with *C. elegans*, genetic selection sys-

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tems using antibiotics in combination with antibiotic resistance genes are a mainstay of molecular genetics research (Chamberlin, 2010). These flexible tools allow stringent, conditional selection of experimentally manipulated individuals. Such a system might also facilitate rapid progress with functional genomics of schistosomes.

Here we investigated drug selection of transgenic schistosomes in order to provide a means to enrich for populations of transgenic worms in virion-exposed parasites. We determined that schistosomes were sensitive in culture to the aminoglycoside antibiotic geneticin (=G418), at doses similar to those reported for other eukaryotes including mammalian cell lines and free living nematodes (Tavoloni, 1997; Yallop and Svendsen, 2001; Giordano-Santini et al., 2010). Schistosomes that were transduced by retrovirus encoding *neoR*, the gene encoding resistance to neomycin, were rescued in culture from G418 toxicity, and the retroviral transgene copy number was enriched in comparison to transgenic schistosomes cultured in the absence of the antibiotic.

2. Materials and methods

2.1. Schistosomes

Biomphalaria glabrata snails infected with *S. mansoni* were supplied by Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD, USA. Schistosomules were obtained from cercariae released from the snails. Cercariae were concentrated by centrifugation (980 g/15 min) and washed in DMEM supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 500 ng/ml amphotericin B and 10 mM HEPES (wash medium). Cercarial tails were removed by 20 passes through a 22 G emulsifying needle, after which schistosomule bodies were separated from tails by Percoll gradient centrifugation (Lazdins et al., 1982). Schistosomula were cultured at 37 °C under 5% CO_2 in Basch's medium (Mann et al., 2010).

2.2. Establishing a G418/geneticin dose–response curve for schistosomules

Schistosomules were transferred to 8 μm mesh polyethylene terephthalate insert membranes and holders (BD Biosciences, USA) inserted into 24-well tissue culture plates. Schistosomules, 500–1,000 per well, were cultured in 125, 250, 500 or 1,000 μg/ml G418 (Geneticin, Invitrogen, USA) or without G418 in Basch's medium for 10 days. Media were changed every second day. In some cases, the detergent Triton X-100 was included at 0.1% or 0.5% (Semple et al., 2010).

2.3. Assessment of viability of schistosomules

Viability of schistosomules was monitored and scored visually. To accomplish this, four or more micrographs of the schistosomules were taken on days 2, 4, 6, 8 and 10 of culture of each treatment condition using a Zeiss Axio Observer A.1 inverted microscope fitted with a 10× magnification objective lens and a digital camera (AxioCam ICc3, Zeiss, Germany). Manipulation of digital images was undertaken with the AxioVision release 4.6.3 software (Zeiss). Each of the micrographs for each G418 concentration recorded non-overlapping regions of the culture plates.

To score the effect of G418 on cultured schistosomes, 150–200 schistosomules for each treatment and time-point group were scored using non-overlapping micrographs. Schistosomules were scored as either live or dead, based on appearance (Clegg and Smithers, 1972; Cottrell et al., 1989). Fig. 1 presents representative images of live and dead schistosomules. Live worms were defined as light or lighter in colour, usually with obvious internal organs including the nascent gut and frequently with an elongate, vermiform appearance (Fig. 1A and B). By contrast, dead schistosomules were defined as dark coloured worms (brown or black), with a granular appearance and often with a more compact, rounded appearance rather than elongate (Fig. 1C) (Cottrell et al., 1989). In some of experiments with G418, aliquots of schistosomules were removed from the culture and incubated in the vital dye Trypan blue (Invitrogen) at 0.1% in PBS for 10 min at 37 °C, 5% CO_2, after which they were scored as dead if stained blue or live if not stained and/or stained weakly and still moving (Fig. 1D and Supplementary Fig. S1) (Cottrell et al., 1989; Gold, 1997). In a similar fashion, in some experiments aliquots of schistosomules were removed from the culture and incubated in the fluorophores fluorescein diacetate (FDA), which stains live schistosomules, and propidium iodide (PI), which stains dead schistosomules, using methods reported by Peak et al. (2010). Assessing viability by (i) visual monitoring of standard cultures, (ii) Trypan blue, or (iii) differential staining by FDA/PI of aliquots of cultured schistosomules, all gave similar results (Fig. 1, Supplementary Fig. S1). Consequently, we employed visual inspection since this procedure allowed maintenance in culture of the entire populations, which could be monitored repeatedly by capturing several micrographs (10× objective) of non-overlapping fields every 2 days.

2.4. Transduction of schistosomules with pseudotyped virions

Vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped murine leukaemia virus (MLV) virions were produced in GP2-293 cells transfected with retroviral constructs, pLNHX_SmAct-Luc or pLNHXΔD70, both of which carry the *neoR* antibiotic resistance gene (Kines et al., 2008; Suttiprapa et al., in press-b). Viral titres were determined using two complementary approaches, a functional (biological) and second a quantitative real-time PCR (qRT-PCR; Retro-X™ qRT-PCR Titration Kit, Clontech, USA) (Mann et al., 2011; Rinaldi et al., 2011). Two days after transformation from cercariae, schistosomules were transduced with virions. Briefly, ~10,000 schistosomules were exposed to virions in 1 ml of medium, 8 μg/ml of polybrene (Sigma–Aldrich); two virion titres were employed, 8×10^5 colony forming units (cfu) (~10^8 virions estimated by qPCR and 2.4×10^6 cfu (~10^9 virions) (Mann et al., 2011). Schistosomules exposed to polybrene but without virions were included as controls. Schistosomes were incubated with the virions for 18 h at 37 °C, 5% CO_2, washed, divided into groups and transferred into media containing G418 at 0, 125, 250, 500 or 1,000 μg/ml. Media including G418 were replaced every second day for 10 days. Micrographs of the schistosomes were collected on days 1, 4, 6, 8 and 10. In addition, aliquots of schistosomules were removed at days 6 and 10 and stored as wet pellets at −80 °C for analysis of transgene copy number and expression.

2.5. Estimation of the transgene copy number

Genomic DNAs (gDNAs) were extracted from virion transduced and control schistosomules and the concentration determined by spectrophotometer. qPCRs were performed using TaqMan probes and primers specific for neomycin phosphotransferase II (*neoR*) (forward primer, 5′-GGA GAG GCT ATT CGG CTA TGA C-3′; reverse primer, 5′-CGG ACA GGT CGG TCT TGA C-3′; probe, 5′-/56-FAM/CTG CTC TGA TGC CGC CGT GTT CCG /3IABlk_FQ/-3′). Reactions were performed using 200 ng of template gDNA in 20 μl of Perfecta qPCR FastMix, UNG (Quanta Bioscience, USA) and a primer-probe set. qPCRs were performed in triplicate, with a denaturation step at 95 °C of 3 min followed by 40 cycles of 30 s at 95 °C and 30 s at 55 °C, in a thermal cycler (iCycler, Bio-Rad, USA) fitted with a real time detector (iQ5, Bio-Rad). Absolute quantification was