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Effects of *Schistosoma mansoni* worms and eggs on circulating cholesterol and liver lipids in mice

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ABSTRACT

It has previously been shown that experimental infections of the parasitic trematode *Schistosoma mansoni*, the adult worms of which reside in the blood stream of the mammalian host, significantly reduced atherogenesis in apolipoprotein E gene knockout (apoE$^{-/-}$) mice. These effects occurred in tandem with a lowering of serum total cholesterol levels in both apoE$^{-/-}$ and random-bred laboratory mice and a beneficial increase in the proportion of HDL to LDL cholesterol. To better understand how the parasitic infections induce these effects we have here investigated the involvement of adult worms and their eggs on lipids in the host. Our results indicate that the serum cholesterol-lowering effect is mediated by factors released from *S. mansoni* eggs, while the presence of adult worms seemed to have had little or no effect. It was also observed that high levels of lipids, particularly triacylglycerols and cholesteryl esters, present in the uninfected livers of both random-bred and apoE$^{-/-}$ mice fed a high-fat diet were not present in livers of the schistosome-infected mice.

1. Introduction

Schistosoma mansoni is a trematode parasite of humans. The male and female adult worms live for many years [1], residing within the mesenteric veins and capillaries of their hosts. Our previous investigations into the possibility that this long-term host–parasite relationship may give some benefit to the host showed that atherogenesis was reduced by approximately 50% compared with uninfected controls in apoE$^{-/-}$ mice given low-intensity, chronic experimental infections of *S. mansoni* [2]. This effect was associated with a significant reduction in serum total cholesterol in the apoE$^{-/-}$ mice, while a highly significant lowering of serum cholesterol levels and a beneficial increase in the proportion of HDL to LDL cholesterol was found in *S. mansoni*-infected, random-bred laboratory mice fed a high-fat diet, when compared with uninfected control mice fed the same diet.

The *S. mansoni*-induced reduction in serum total cholesterol of random-bred high-fat diet-fed mice commenced at a time that coincided with the production of eggs [2]. The cholesterol-lowering effect may therefore be a result of factors on, or released by schistosome eggs, by mature worms, or by both. High levels of circulating cholesterol are one of the most important risk factors for heart disease in the developed world and the importance of the continuous search for compounds with lipid-lowering properties cannot therefore be overstated. To gain further insight into the serum cholesterol-lowering effect of *S. mansoni* infection, and as a step towards more exact identification of the parasite-derived factors responsible, we exploited the dioecious nature of the parasite to examine the effects of either single-sex worm-alone schistosome infections or intraperitoneal injections of schistosome eggs or egg-derived factors.

2. Materials and methods

2.1. Mice

ApoE$^{-/-}$ mice were obtained from a breeding colony at the Bristol Heart Institute, University of Bristol, UK. Random-bred Theiler's Original (TO) strain mice were used as a 'normal' mouse for the study of infection-induced cholesterol-lowering and were obtained from Bantin & Kingman (Hull, UK).

Unless otherwise stated mice were fed standard laboratory diet (RM3; Special Diet Services, Wincham, Cheshire, UK) *ad libitum*. High-fat diet (High-Fat Diet M) was also purchased from Special Diet Services and contained 0.15% cholesterol and a further 21% lipid in the form of lard.

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2.2. Schistosome infections

An isolate of *S. mansoni* from Puerto Rico was used throughout this work. Infections of cercariae were applied percutaneously as described [3].

All procedures on experimental animals were performed strictly under the provision of licences granted under the UK Animals (Scientific Procedures) Act 1986. All mice were checked for health daily. Results from animals displaying overt symptoms of schistosomiasis (anaemia, weight loss, cachexia, bloody diarrhoea) were excluded.

2.3. Worm-alone, single-sex schistosome infections

Male and female *S. mansoni* cercariae were obtained by infecting individual *Biomphalaria glabrata* snails with single male or female miracidia as previously described [4]. Cercariae of the required sex were later collected from the host snails for use in experimental mouse infections.

2.4. Preparation of S. mansoni eggs and egg antigens

Intact schistosome eggs were extracted from tissues (liver and intestines) of patently infected mice by homogenization, repeated filtrations and gravity-induced sedimentations in 1.8% NaCl solution [5].

Aqueous solutions of *S. mansoni* soluble egg antigens (SmSEA) [5] were produced by mechanical homogenization of schistosome eggs suspended in a small volume of phosphate buffered saline (PBS pH 7.2) over ice. The homogenate was centrifuged in a microfuge at 10,000 rpm for 5 min and the supernatant transferred to a new tube. The remaining pellet was resuspended in PBS and centrifuged again. Both supernatants were combined and centrifuged at 10,000 rpm for 20 min. Volumes of PBS used were such that the BSA-equivalent protein concentrations in the resulting supernatants were 10–15 mg/ml. Aliquots of the final supernatant (SmSEA) were then stored at −80 °C.

S. mansoni egg culture supernatant (SmECS) was prepared essentially as described by Ashton et al. [6]. Batches of approximately 0.5 million *S. mansoni* eggs were washed twice in cell culture medium (RPMI-1640 containing 300 U/ml penicillin, 300 μg/ml streptomycin and 50 μg/ml gentamycin). The eggs were then resuspended in 60 ml of prepared medium and 5 ml aliquots placed in the wells of 6-well culture plates (Costar; Corning Inc., NY, USA) and incubated at 37 °C. Cultures were checked daily for contamination and egg viability. The supernatant was harvested after 48 h. SmECS was concentrated approximately 15-fold to 4 ml using centrifugal devices (Microsep; Pall Life Sciences, Portsmouth, UK) with a 1 kDa cut-off filter. The protein concentration was determined, and the quality of individual batches of SmECS was confirmed in Western immunoblots by probing with rabbit sera raised against purified *S. mansoni* egg antigens alpha-1, omega-1, and k-5 [7], and by protein stain (Protogold; BBS Scientific).

2.5. Blood cholesterol determinations

Mice were fasted for 15 h overnight prior to the withdrawal of blood from a superficial vein. After being allowed to clot at 37 °C for 1 h and at 4 °C for 4 h blood samples were centrifuged (Micro Centaur, MSE Scientific Instruments, Crawley, UK) and the serum stored at −20 °C until required. Serum total cholesterol was estimated using assay kits from Sigma ("Infinity" cholesterol reagent-procedure 401-500P, cholesterol calibrators C0534; Poole, UK) in accordance with the manufacturer's instructions.

2.6. Thin layer chromatography

Neutral lipids were extracted from 50 mg mouse liver tissue by homogenization in 0.5 ml of a mixture of chloroform:methanol (2:1). The supernatant was removed after centrifugation in a microfuge at 13,000 rpm for 6 min and the supernatant extracted. The extraction in chloroform:methanol was repeated twice. To 1.5 ml of resulting supernatant was added 0.35 ml of 0.88% (w/v) KCl in water. After vortexing, the lipid-containing layer was removed and stored in glass containers at −20 °C. 10 μl aliquots of each extracted liver sample and of a mixture of neutral lipid standards (Sigma, Poole, UK) were applied to the origin on a 20 cm × 20 cm silica 60 TLC plate (Merck Ltd., Lutterworth, UK). The plate was developed vertically with a petroleum ether:diethyl ether:acetic acid (80:20:1) mobile phase in a chromatography tank with an atmosphere pre-saturated with the solvent mixture. The developed plate was stained with Sudan black B and de-stained in a 1:1 mixture of ethanol and water.

The density of each spot was recorded (Gel-Analysis software, Kodak Scientific) and converted into quantities of lipid (μg per 100 mg of liver tissue sample) using calibration curves constructed for each of the neutral lipid standards.

2.7. Histology

Liver samples were fixed in 10% formalin for 24 h after which they were dehydrated in a graded series of ethanol solutions (25%, 50%, 75% and 100% ethanol) at 4 °C over a period of 2 days. The samples were then placed in a 1:1 mixture of ethanol and resin infiltration solution on a rotator overnight and placed in 100% infiltration solution for 2–5 days on a rotator. 4 μm sections were cut, stained in haematoxylin and eosin and mounted using DPX (BDH Laboratory Supplies, Poole, UK.) and a coverslip.

For histochemical detection of lipids, blocks of liver tissue (approximately 5 mm) were cut from the livers of freshly perfused mice and immediately frozen in liquid nitrogen for sectioning in a cryostat at −20 °C. Sections of liver tissue (20 μm) were fixed for 10 min in 10% phosphate buffered formalin then dipped in 70% alcohol before staining in a 0.7% solution of Sudan black B for 1 h. The sections were again placed in 70% alcohol and washed under running water before mounting for microscopic examination.

2.8. Statistics

The Student's *t*-test was used to compare two experimental groups after testing for normality (Bartlett's test for equal variances). One-way ANOVA and the Tukey post-test were applied to the comparison of more than two experimental groups after testing for normality. The statistical tests used in individual experiments are indicated in Section 3.

All statistical tests were carried out using GraphPad InStat software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Single-sex worm-alone infections

Experiments to investigate the effect of *S. mansoni* worms on host serum cholesterol were carried out in random-bred mice. Thus, 28 TO strain male mice were placed on high-fat diet (HFD) and an equal number were left on a normal (ND) diet. Two weeks later 10 mice from each of the dietary groups were given a percutaneous infection of 250 male *S. mansoni* cercariae, and a further 10 mice from each of the dietary groups were infected with 250 female *S. mansoni* cercariae. The remaining animals were left as uninfected controls for each diet. Sixteen weeks after infection the infected