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Penetration of human skin by the cercariae of *Schistosoma mansoni*: an investigation of the effect of multiple cercarial applications

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Abstract

It has previously been postulated that L-arginine emitted by penetrating *Schistosoma mansoni* cercariae serves as an intraspecific signal guiding other cercariae to the penetration site. It was suggested that penetrating in groups offers a selective advantage. If this hypothesis is correct and group penetration at one site on the host offers an advantage, it would follow that at such a site, successive groups of cercariae would be able to penetrate skin in either greater numbers or at a faster rate. This prediction was tested by the use of an *in vitro* model of cercarial penetration based on the Franz cell and using human skin. It was demonstrated that there was no increase in the percentage of cercariae able to penetrate the skin with subsequent exposures. Consequently, it seems unlikely that the release of L-arginine by cercariae during penetration could have evolved as a specific orientation system based on a selective advantage offered by group penetration.

Introduction

Schistosomiasis is endemic in 74 countries and it is estimated that 200 million people are infected, with a further 500–600 million at risk (Chitsulo *et al.*, 2000). Infection occurs when larvae (cercariae) of the parasite penetrate human skin during contact with infected fresh water. In the past, either non-human laboratory model hosts or artificial systems have been used to investigate penetration (Wilson & Lawson, 1980; Wilson, 1987; Wilson *et al.*, 1990; Fusco *et al.*, 1993). Results from these experiments cannot, however, be directly extrapolated to the events that occur on the surface of human skin, because the skin of other mammals is very different from human skin in terms of anatomy, physiology, hairiness, epidermal/dermal thickness and the chemical nature of surface secretions (Monteiro-Riviere, 1996; Behl *et al.*, 1990). In addition, although artificial skin equivalents constructed partially or wholly from human skin cells do provide a physical model of skin, their basic properties are very different (Behl *et al.*, 1993).

The entry of schistosome cercariae into the skin of the mammalian host has been described as having three phases: attachment, creeping over the surface of the skin to locate a site of entry and penetration into the epidermis (Haas & Schmitt, 1982). In the case of *Schistosoma mansoni*, attachment to the host is reported to be stimulated in response to L-arginine (Granzer & Haas, 1986). The fact that the acetabular gland secretions of cercariae contain arginine (Stirwalt & Walters, 1973) has lead to the hypothesis that L-arginine, emitted by penetrating cercariae, serves as an intraspecific signal guiding other cercariae to the penetration site (Haas, 1994). It has been further hypothesized that penetrating in groups offers a selective advantage. Such a theory is supported by Griffiths (1953) who suggested that when large numbers of cercariae are applied to a limited area of skin, the process of penetration might be facilitated by some early-penetrating cercariae preparing the way for others

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following in their wake. It was observed that during the penetration of large numbers of cercariae into mouse skin, the cercariae could be seen to utilize the tunnels left in the skin by previous penetrants. Consequently, cercariae from multiple penetrations were reported to reach the dermis far more rapidly than those penetrating the skin in small numbers. Furthermore, Standen (1953) reported that when large numbers of cercariae penetrate a small area of mouse skin, the multiple release of the parasite's proteolytic secretions results in significant damage to the skin and a peeling away of the stratum corneum and epidermis. The stratum corneum and epidermis are significant physical and chemical barriers to cercariae (Fukuyama *et al.*, 1983), and their removal could offer an advantage to the invading larvae.

If the hypothesis proposed by Haas (1994) is correct and penetrating in groups at one site on the host offers a selective advantage, it would follow that at this site on the host, successive groups of cercariae would be able to penetrate skin either in greater total numbers or at an initially faster rate, or both. In the present study this prediction was tested by the use of an *in vitro* model of cercarial penetration based on the Franz cell and using human skin (Bartlett *et al.*, 2000).

Materials and methods

Skin and Franz cell preparation

Human skin was obtained from female patients, aged between 25 and 50 years, undergoing elective abdominoplasty. Excised full thickness skin was frozen at −20°C within 2 h of surgery and was defrosted prior to use at 4°C overnight. (Such thawed, non-viable skin could have different properties from living human skin in relation to the outward diffusion of small water-soluble molecules like arginine. The impact of this possible difference on the results of the present study is undetermined.)

The skin samples were prepared as previously described (Brown *et al.*, 1995). The skin surface was thoroughly washed with water to remove any surgical antiseptic and the subcutaneous fat was removed by careful dissection, taking care not to puncture the surface of the skin. The skin was cut into circular, 3 cm diameter, pieces and each piece mounted in a Franz cell with the epidermis uppermost. The receptor well was filled immediately with tissue culture medium (CO_2 independent medium with 10% FCS and 1% Antibiotic/Antimycotic solution, Gibco). Cells set up with inert, latex rubber membranes held in place with Teflon 'o-rings' were used as controls for the method of recovery of cercariae. Each Franz cell was submerged in a circulating water bath at 37°C and the water level was maintained just below the lip of the receptor well, thus ensuring that the skin surface temperature was about 32°C. The system was allowed to equilibrate for 1 h before use, in order to ensure complete hydration and temperature stability of the skin.

Parasites

A Puerto Rican strain of *S. mansoni* was maintained in *Biomphalaria glabrata* snails and NMRI strain, female laboratory mice as described by Standen (1949), with the modification that *S. mansoni* eggs were obtained from livers of infected mice. Infected snails were randomly separated into five opaque containers to prevent them being exposed to any light. Then cercarial populations were obtained by exposing the snails, in filtered water at 28°C, to strong light and allowing them to shed cercariae for 30 min. The mean cercarial density was determined from five 1 ml samples of each suspension of cercariae fixed and stained with Lugol's iodine. Each suspension was then diluted to give about 20 cercariae per ml and 5 ml of suspension was applied to skin in Franz cells as described below.

Penetration of cercariae

Infection experiments were carried out with skin receiving five sequential applications of cercariae with a maximum interval of 10 min between each exposure. Two time periods were chosen for the duration of exposure of skin to cercariae. The total number of cercariae able to become irreversibly attached to the skin at each exposure was determined by allowing the cercariae to remain in contact with the skin for 20 min, since all cercariae able to penetrate the skin in this model system would have done so by this time (Bartlett *et al.*, 2000). Changes in the initial rate of cercarial attachment with each exposure were investigated by repeating the experiment but allowing the cercariae to remain in contact with the skin only for 5 min. Controls were set up to compare the numbers of each individual cercarial population able to attach to previously unexposed skin at each time point. To control for the action of hydration on cercarial attachment rates, water was applied to control cells in the place of multiple applications of cercariae. The regime of water and cercarial application to cells is shown in table 1.

At the end of the 5 or 20 min periods of exposure, each cercarial suspension was removed from the upper well with vigorous pipetting, the skin was then washed twice with a total of 10 ml of distilled water to remove any cercariae that were not firmly attached to the skin. The removed cercariae were fixed and stained with Lugol's iodine and the numbers recovered counted.

Histology

At the end of the study the skin samples were removed from the Franz cells and fixed in Carnoy's fluid overnight. The samples were then transferred to 70% ethanol, dehydrated and embedded in paraffin wax. Sections were cut at 8 μm, stained with Ehrlich's haematoxylin/eosin and mounted in XAM synthetic resin.

Statistical analysis

The mean percentages of cercariae recovered from the experimental and control cells were calculated and the difference between them expressed graphically. Statistical analysis was carried out on the arcsine^{-1} values ($\sqrt{x}$ sine^{-1}) of the percentage data to ensure normality of distribution. ANOVA was used to compare subsequent exposures. Differences between the experimental and control samples were examined using a paired t-test.

Table 1. Application of cercariae to test and control Franz cells.

	Exposure				
Sample (n = 10)	1st	2nd	3rd	4th	5th
Experimental cells	Cercariae	Cercariae	Cercariae	Cercariae	Cercariae
Control 1	Cercariae	–	–	–	–
Control 2	Water	Cercariae	–	–	–
Control 3	Water	Water	Cercariae	–	–
Control 4	Water	Water	Water	Cercariae	–
Control 5	Water	Water	Water	Water	Cercariae
Inert membrane	Cercariae	Cercariae	Cercariae	Cercariae	Cercariae

Results

Franz cell

The mean percentage of cercariae recovered from the inert membranes was 99% (data not shown), demonstrating that the washing method recovers unattached cercariae reliably from the Franz cell. The mean percentages (± SD) of cercariae recovered from control and experimental cells, i.e. those not irreversibly attached to or penetrating the skin, are shown in table 2.

Figure 1 shows the differences from the appropriate controls, of the mean percentage (± SD) of cercariae penetrating the skin at 5 and 20 min. When the number of cercariae recovered from the control and experimental Franz cells was compared using an overall paired t-test there was no statistically significant difference between the results either for those exposed for 5 min ($P = 0.684$) or 20 min ($P = 0.654$). There was also no statistically significant difference between the percentage of cercariae recovered separately from exposures 1–5 at each of the time points as determined by ANOVA (5 min $P = 0.684$, 20 min $P = 0.875$).

Histology

Histological examination showed that within the experimental skin samples given five successive exposures to cercariae, there were very large numbers of cercariae and/or transforming schistosomula. Cercariae were attached to the stratum corneum, within the epidermis or in immediate sub-epidermal locations, as well as deeper in the dermis. Some images showed that cercariae were penetrating the skin in close proximity to each other, however, there was no indication of large numbers of cercariae sharing an entry site or a subsequent tunnel. Despite the application of numerous cercariae, there was no evidence of peeling back of the epidermal layer. However, there was localized damage to the stratum corneum at sites of cercarial entry. There was also evidence in a number of places that cercariae, tunnelling horizontally beneath the stratum corneum, led to its separation from the underlying epidermis.

Table 2. The attachment of cercariae to skin in the control and experimental Franz cells in successive exposures.

		Mean % cercariae recovered (± SD)	
Time	Exposure	Control skin	Experimental skin
5 min	1	42 ± 17	38 ± 10
	2	71 ± 17	73 ± 20
	3	44 ± 1	44 ± 7
	4	49 ± 4	48 ± 7
	5	47 ± 2	48 ± 7
20 min	1	25 ± 13	25 ± 13
	2	18 ± 4	25 ± 10
	3	40 ± 19	40 ± 14
	4	34 ± 5	32 ± 7
	5	27 ± 3	25 ± 4

Discussion

The aim of this investigation was to determine if the previous penetration of a section of human skin by cercariae increases either the rate of attachment of cercariae or the total number able to penetrate in a subsequent exposure. We have assumed that any cercariae which could not be removed from the skin by extensive washing were irreversibly attached to or had penetrated the skin as demonstrated by Bartlett *et al.* (2000). If successive exposures of cercariae were able to penetrate in increasing numbers there would have been a progressive reduction in the percentage of cercariae recovered after 20 min: no such change was observed (fig. 1a). Figure 1b shows there were also no changes in the percentages of cercariae recovered after 5 min exposure, demonstrating that there was no increase in the initial rate at which the cercariae penetrate no matter how many previous exposures had occurred. This would indicate that if group penetration were to occur it does not offer any advantage in the penetration process.

The histological examination of the skin after multiple exposures to cercariae also would not seem to support the theory of advantage to group penetration. In the present study there was no evidence of the peeling back of the stratum corneum and epidermis as reported by Standen (1953), although there was disturbance of the stratum corneum at the point of cercarial entry and in places, cercarial penetration was associated with a separation of the stratum corneum from the underlying epidermis. Although it is possible that this separation was an artefact of the histological procedure (Gordon & Griffiths, 1951; Wilson & Lawson, 1980), Bruce *et al.* (1970) showed that such changes also occur as a result of cell damage. Such

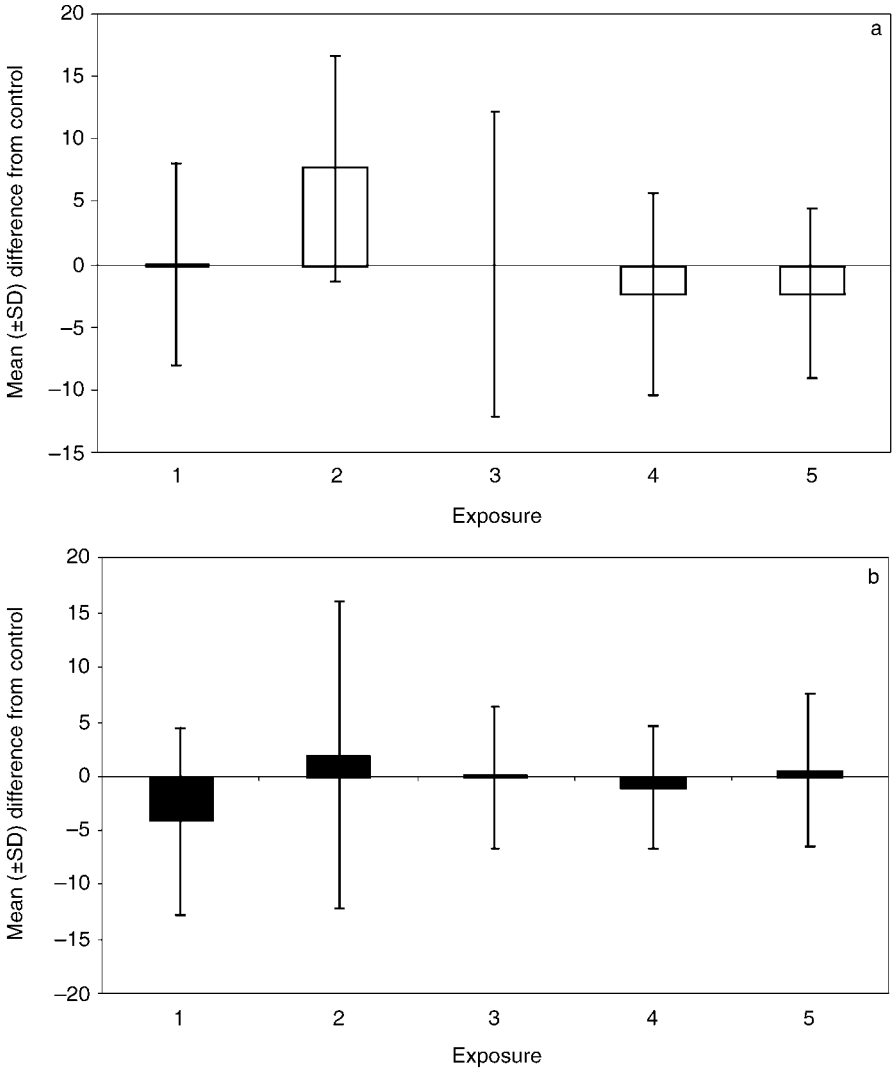

Fig. 1. The mean difference (± SD) between % cercariae recovered from control and experimental Franz cells at (a) 20 min (□) and (b) 5 min (■).

an effect could be due to the lytic action of secretions of the cercariae and/or schistosomula.

The development of an intraspecific signal guiding cercariae to a particular penetration site is not supported by the results described here and seems improbable, especially when it is considered that in infected waters cercariae are widely dispersed. For example, at sites in St Lucia, Upatham & Sturrock (1973) found cercariae at densities from 0.01 to 150 per litre of water. In the present study, the skin was subjected to the equivalent of 20,000 cercariae per litre at each exposure. If group-assisted penetration did not occur under the conditions described in the present study, it is unlikely to happen in the field where a much smaller number of cercariae will be invading a host at any one time. Consequently, it seems unlikely that a selective advantage offered by group penetration could have given rise to the evolution of a specific orientation system based on the cercarial production of L-arginine (Haas, 1994). Obviously, the present study leaves open the question of why schistosome cercariae appear to respond to L-arginine and this warrants further investigation as this amino acid is clearly not a specific marker for human skin.

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References

Bartlett, A., Brown, M., Marriott, C. & Whitfield, P.J. (2000) The infection of human skin by schistosome cercariae: studies using Franz cells. *Parasitology* **121**, 49–54.

Behl, C.R., Kumar, S., Patel, S. & Piemontese, P. (1990) Choice membranes for *in vitro* skin uptake studies. pp. 1–23 *in* Kemppainen, B.W. & Reifenrath, W.G. (*Eds*) *Methods for skin absorption*. Boston, CRC Press.

Behl, C.R., Metha, D.B., Malick, A.W., Patel, S.B., Char, H. & Piemontese, D. (1993) *In vivo* and *in vitro* skin uptake and permeation studies. pp. 225–259 *in* Shah, V.P. & Maibach, H.I. (*Eds*) *Topical drug bioavalability, bioequivalance and penetration*. New York, Plenum Press.

Brown, M.B., Marriott, C. & Martin, G.P. (1995) The effect of hyaluronan on the *in vitro* deposition of diclofenac within the skin. *International Journal of Tissue Reactions* **17**, 133–140.

Bruce, J.I., Pezzlo, F., McCarthy, J.E. & Yajima, Y. (1970) Migration of *Schistosoma mansoni* through mouse tissue: ultrastructure of host tissue and integument of migrating larvae following cercarial penetration. *American Journal of Tropical Medicine and Hygiene* **19**, 959–981.

Chitsulo, L., Engels, D., Montresor, A. & Savioli, L. (2000) The global status of schistosomiasis and its control. *Acta Tropica* **77**, 41–51.

Fukuyma, K., Tzeng, S., Mckerrow, J. & Epstein, W.L. (1983) The epidermal barrier to *Schistosoma mansoni* infection. *Current Problems in Dermatology* **11**, 185–193.

Fusco, A.C., Cassioppi, L., Salafsky, B. & Shibuya, T. (1993) Penetration of *Schistosoma mansoni* cercariae into a living skin equivalent. *Journal of Parasitology* **79**, 444–448.

Gordon, R.M. & Griffiths, R.B. (1951) Observations on the means by which the cercariae of *Schistosoma mansoni* penetrate mammalian skin, together with an account of certain morphological changes in the newly penetrated larvae. *Annals of Tropical Medicine and Parasitology* **45**, 227–434.

Granzer, M. & Haas, W. (1986) The chemical stimuli of human skin surface for the attachment response of *Schistosoma mansoni* cercariae. *International Journal for Parasitology* **16**, 575–579.

Griffiths, R.B. (1953) Further observations on the penetration of mammalian skin by the cercariae of *Schistosoma mansoni*, with special reference to the effects of mass invasion. *Annals of Tropical Medicine and Parasitology* **47**, 86–94.

Haas, W. (1994) Physiological analysis of host-finding behaviour in trematode cercariae: adaptations for transmission success. *Parasitology* **109**, S15–S29.

Haas, W. & Schmitt, R. (1982) Characterisation of chemical stimuli for the penetration of *Schistosoma mansoni* cercariae. *Zeitschrift für Parasitenkunde* **66**, 293–307.

Monteiro-Riviere, N.A. (1996) Anatomical factors effecting barrier function. pp. 3–18 *in* Murzulli, F.N. & Macibach, H.I. (*Eds*) *Dermatotoxicity*. 5th edn. London, Taylor and Francis.

Standen, O.D. (1949) Experimental schistosomiasis II: maintenance of *Schistosoma mansoni* in the laboratory, with some notes on experimental infection with *S. haematobium*. *Annals of Tropical Medicine and Parasitology* **43**, 268–283.

Standen, O.D. (1953) The penetration of cercariae of *Schistosoma mansoni* into the skin and lymphatics of the mouse. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **46**, 384–398.

Stirwalt, M.A. & Walters, M. (1973) *Schistosoma mansoni*: histochemical analysis of the postacetabular gland secretion of cercariae. *Experimental Parasitology* **33**, 56–72.

Upatham, E.S. & Sturrock, R.F. (1973) Studies on the effects of cercarial concentration and length of exposure on the infection of mice by *Schistosoma mansoni*. *Parasitology* **67**, 219–228.

Wilson, R.A. (1987) Cercariae to liver worm: development and migration in the mammalian host. pp. 115–146 *in* Rollinson, D. & Simpson, A.J.G. (*Eds*) *The biology of schistosomes: from genes to latrines*. London, Academic Press.

Wilson, R.A. & Lawson, J. (1980) An examination of the skin phase of schistosome migration using a hamster cheek pouch preparation. *Parasitology* **80**, 257–266.

Wilson, R.A., Coulson, P.S. & Sturrock, R.F. (1990) Schistosome migration in primates: a study in the olive baboon (*Papio anubis*). *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84**, 80–83.

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