The infection of human skin by schistosome cercariae: studies using Franz cells

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SUMMARY

Franz cells (2-chambered, air/fluid phase static diffusion devices, previously used for the study of drugs across viable human skin) are utilized for the first time to investigate the process of infection of human skin by *Schistosoma mansoni* cercariae. Skin obtained from cosmetic surgery sources was used in the Franz cells to describe the temporal dynamics of the early interaction of cercariae with skin. At 38 °C, about 50 % of cercariae applied in water to the epidermal surface of the skin were irreversibly attached within 1 min and after 5 min about 85 % were similarly irrecoverable. The technique also provides the means of following the early penetration path of cercariae by histological methods. Franz cell results on the dynamics of attachment/early penetration have been compared with those obtained using artificial skin equivalents and non-human mammalian skin models in the context of the physical and chemical differences between these systems and viable human skin. It is concluded that Franz cells provide a convenient system for directly investigating the early phases of *S. mansoni* cercariae interaction with human skin.

Key words: *Schistosoma mansoni*, cercariae, human skin, Franz cells.

INTRODUCTION

Little directly-obtained information is available on the processes by which schistosome cercariae recognize, adhere to and eventually penetrate human skin to establish the infections that result in schistosomiasis. The great majority of attempts to understand this crucial sequence of events have been based on experiments using either non-human model hosts or artificial skin systems. Some experiments (see, for example, Haas *et al*. 1997) have studied schistosome cercarial behaviour in the absence of skin, but in the presence of individual skin-derived chemicals, in an attempt to understand chemical triggering of cercarial secretions during attachment and penetration. Wilson (1987) has reviewed studies which have used laboratory rodents as the targets for schistosome cercarial infections whilst Wilson *et al*. (1990) have described the use of olive baboons (*Papio anubis*) for studies on skin penetration and subsequent parasite migrations within the final host. Fusco *et al*. (1993) have examined the penetration of *Schistosoma mansoni* cercariae into a 'living skin equivalent', consisting of human dermal fibroblasts embedded in a collagen lattice and an epidermal layer consisting of differentiated keratinocytes that form a stratum corneum-like layer.

Underlying the interpretation of all such experiments is an assumption that the results of these studies allow extrapolation to the infection process which actually occurs at the human skin surface. There are compelling reasons, however, for treating this assumption with some scepticism. Studies in dermatology, cutaneous pharmacology, toxicology and percutaneous absorption, have shown that the skin of model mammals like rats, mice and guinea-pigs is very different from human skin in relation to anatomy and physiology, hairiness, stratum corneum, epidermis/dermal thickness and the chemical nature of surface secretions (Behl *et al*. 1990; Montiero-Riviere, 1996).

In addition, although artificial skin equivalents (Basset-Seguin *et al*. 1990; Triglia *et al*. 1991; Slivka *et al*. 1993) constructed partly or wholly from human skin cell types do provide a physical model of skin, they do not mimic its attributes completely (Behl *et al*. 1993). In fact, recent penetration studies demonstrated that a cultured skin alternative was much more permeable to exogenous materials than human cadaver skin (Goodwin, Michniak & Creek, 1997). Even with cadaver skin, problems can result due to deterioration of the skin viability between death and sample harvest (Bronaugh, Stewart & Simon, 1986), whilst the individual, the regional site, age and disease state of the skin can all affect the permeability (Wester & Maibach, 1992).

In the context of these interpretational difficulties, there is a clear need for re-examining the seldom-used experimental approaches which do expose

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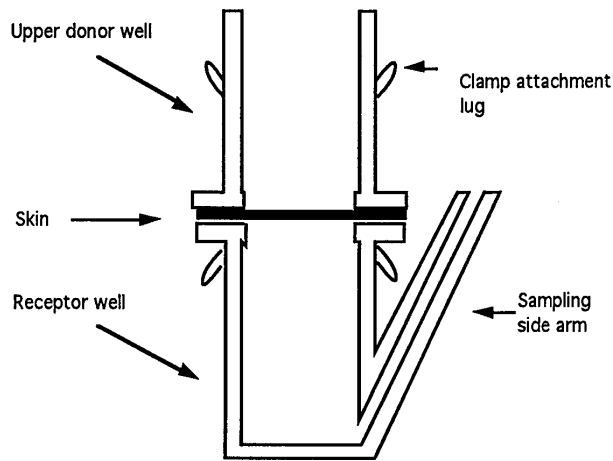
Fig. 1. The Franz cell.

viable human skin to schistosome cercariae. The pioneering study of Stirewalt (1957) examined the behaviour of *S. mansoni* cercariae on a variety of skin surfaces by direct light microscopical observation. The skins included those of nude mice, C3H mice, rats, golden hamsters, macaque monkeys and humans. The human skin in these experiments came from 3 sources, the dorsum of a toe after freezing and thawing, the abdomen of a cadaver 18 h after death and the finger-tip and dorsum of the basal joint of a finger. The experiments on finger skin appear to have been carried out on skin *in situ* on a living human volunteer. These experiments provided useful preliminary information on the surface behaviour and penetration dynamics of the cercariae with respect to human skin but have almost never been directly followed up. In fact, only 1 more recent study seems to have made experimental use of human skin in a similar fashion. Cohen *et al.* (1991) modelled the molecular structure of the *S. mansoni* cercarial serine protease from the amino acid sequence predicted by its cDNA clone, taking advantage of its sequence similarity to the trypsin-like class of proteases. They then used the model to design a series of synthetic inhibitors of the enzyme. These inhibitors were tested against *S. mansoni* cercariae invading human skin obtained from autopsies (6–12 h after death) or from amputation specimens.

The paucity of direct infection studies using human skin is likely to be mainly due to the logistical and ethical difficulties associated with obtaining a regular supply of skin from cadavers or amputations. Studies using human volunteers (Stirewalt, 1957) are unlikely ever to be repeated!

The present study describes a novel and ethical method for investigating the interactions between schistosome cercariae and human skin which overcomes many of the inadequacies of the more indirect approaches. The experiments described here have used viable human skin obtained from cosmetic surgical sources and supported in an air/fluid phase,

static diffusion cell, termed a Franz cell (Franz, 1975). These are 2-chambered glass enclosures which have been used since the mid 1970s for the investigation of drug uptake across both human and animal skin (see Fig. 1). Our experiments consider, for the first time, the schistosome cercarial infection process using this established methodology for work on living human skin. Clegg & Smithers (1972) used a 2-chambered glass apparatus superficially similar to a Franz cell for schistosome cercarial studies. They clamped mouse skin (from which the dermis had been abraded) in their apparatus in order to transform *S. mansoni* cercariae to schistosomula for subsequent *in vitro* cultivation. These workers did not use human skin in their penetration apparatus.

MATERIALS AND METHODS

Parasites

A Puerto Rican strain of *S. mansoni*, originally obtained from the Liverpool School of Tropical Medicine and Hygiene, was routinely maintained in *Biomphalaria glabrata* snails and NMRI strain, female laboratory mice as described by Standen (1949). Cercariae were obtained by exposing snails with patent infections to strong light and allowing them to shed cercariae into filtered water for no more than 2 h. The suspension of cercariae was then separated, a 1 ml aliquot was fixed with Lugol's iodine and the cercariae counted under a low power ($\times 20$) microscope. The volume was adjusted to give a density of 100–150 cercariae/ml of filtered water and 5 replicate counts of 1 ml aliquots were made to estimate the mean number of cercariae/ml.

The Franz cell

The cell consists of 2 circular, glass wells with ground glass rims (Fig. 1). Skin can be clamped between the two wells by means of spring clamps and the lower part of the cell is then filled with tissue culture medium through the side arm, which also enables sampling and medium change.

Preparation of skin

Human skin was obtained from female patients, aged between 25 and 50 years, undergoing elective abdominoplasty. Excised, full thickness skin was either used immediately or frozen at $-20\ ^{\circ}$C within 2 h of surgery and stored until required; it was defrosted at room temperature immediately before use. Full thickness skin, both dermis and epidermis, was prepared as previously described (Brown, Marriott & Martin, 1995). Briefly, the surface was washed extensively with water to remove surgical

Table 1. *Mean percentage of cercariae recovered from upper, donor wells at intervals up to 60 min after their addition to these wells*

Time in min ...	0	1	2·5	5	10	15	20	30	60
Skin	76	51	36	16	14	14		12	13
n =	10	12	13	7	7	7	7	16	8
Inert membrane	76			74		74		79	80
n =	10			10		10		10	10

antiseptic then subcutaneous fat was removed by careful dissection, taking great care not to puncture the surface. The skin was cut into circular, 3 cm diameter, pieces and each piece mounted in a Franz cell with epidermis uppermost. The receptor well was immediately filled with tissue culture medium (CO_2 independent medium with 10 % FCS and 1 % antibiotic/antimycotic solution – GIBCO). Care was taken throughout the experiments to maintain the skin in direct contact with the tissue culture medium excluding any air bubbles by means of the side-arm.

The Franz cell was submerged in a circulating water bath at 38 °C so that the water level was maintained just below the lip of the receptor well: the system was allowed to equilibrate for 1 h before use.

Dynamics of irreversible cercarial attachment to skin

Cells were set up as described and 8 ml of cercarial suspension added to each upper well. At precise time-intervals (between 1 and 60 min subsequently), the cercarial suspensions were stirred to dislodge any cercariae not firmly attached, then removed to counting dishes. Cercariae were fixed with Lugol's iodine and detached heads plus whole cercariae were counted. Control experiments with the same procedures were set up with an inert rubber membrane in place of the skin and a Teflon 'o' ring inserted above the membrane to ensure that there was no leakage from the upper well.

Penetration of schistosomula

Franz cells were set up with skin as described and at least 1000 cercariae in 8 ml were put into each upper well. At 24 h, the tissue culture medium in the receptor wells was removed and transferred to counting dishes which were scanned under low power ($\times 30$) for schistosomula. A third of the cells were dismantled and the skin fixed in Carnoy's fluid for histology. Fresh medium was added to the remaining cultures and the process repeated to give samples at 48 and 72 h.

This was repeated with skin from 4 different patients, 6–9 cells being used each time according to the size of the piece of skin obtained.

Histology

Skin pieces fixed in Carnoy's fluid were 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.

RESULTS

Dynamics of cercarial attachment to skin

The percentage of cercariae recovered and the number of replicates carried out at any one time-point are shown in Table 1.

The means and standard deviations of the percentages recovered from Franz cell upper wells in experiments using skin and inert membranes as controls, are shown in Fig. 2.

Addition, then immediate removal of cercariae from the upper cell, with an inert membrane in place, showed that 15–30 % (mean 24 %) of cercariae were mechanically not recoverable. This value was unchanged throughout 60 min of control investigations.

In contrast, after only 1 min in contact with skin, about 50 % of cercariae were attached firmly and could not be recovered. At 2·5 min, about 65 % were firmly attached and after 5 min about 85 % were firmly attached and had begun to penetrate the skin. The remaining 15 % of the population had not become attached to the skin after 60 min.

Penetration and recovery of schistosomula

Some schistosomula could be found in the receptor wells in each penetration experiment; typically, 2–3 at 24 h, another 5–6 at 48 h and 1–2 more at 72 h, this being the total recovery from 6–9 cells each with at least 1000 cercariae. All those which were found were alive and moving vigorously. Very few schistosomula, however, achieved full-thickness skin penetration. In spite of repeated effort, with manipulation of culture conditions (changing serum levels or using human serum instead of foetal bovine), it was never possible, up to 72 h after cercarial application, to recover more than a total of 0·1 % of the cercariae applied to the surface as emerged schistosomula in the receptor wells.
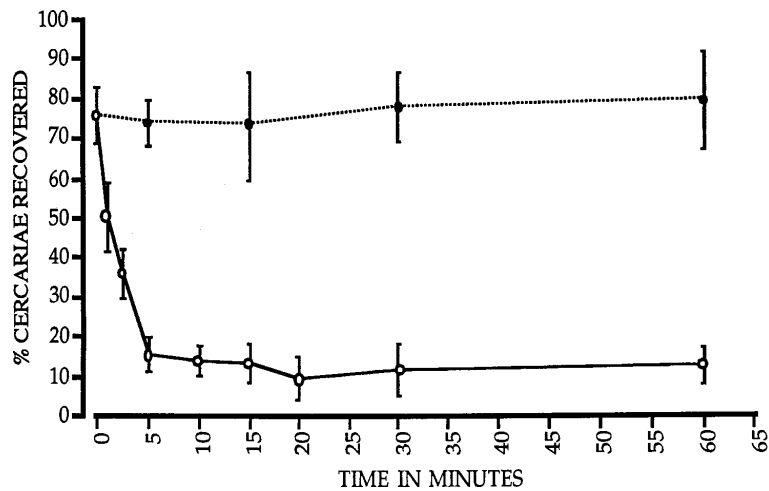
Fig. 2. The means and standard deviations of the percentages of cercariae recovered from the upper wells of Franz cells. (○) Cells with human skin; (●) cells with an inert rubber membrane.
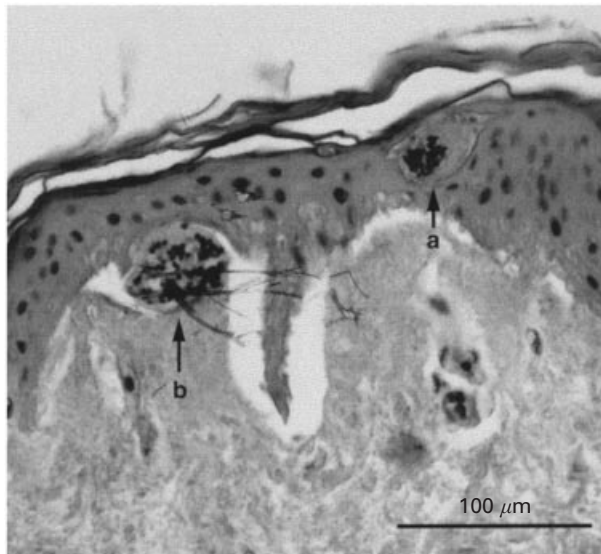

Fig. 3. Schistosomulum penetration after 24 h. H & E stained section showing 2 schistosomula. One (a) has completely penetrated the stratum corneum and is now embedded in the cells of the viable epithelium, the other (b) has completely penetrated the viable epithelium and rests on the basement membrane above the dermis.
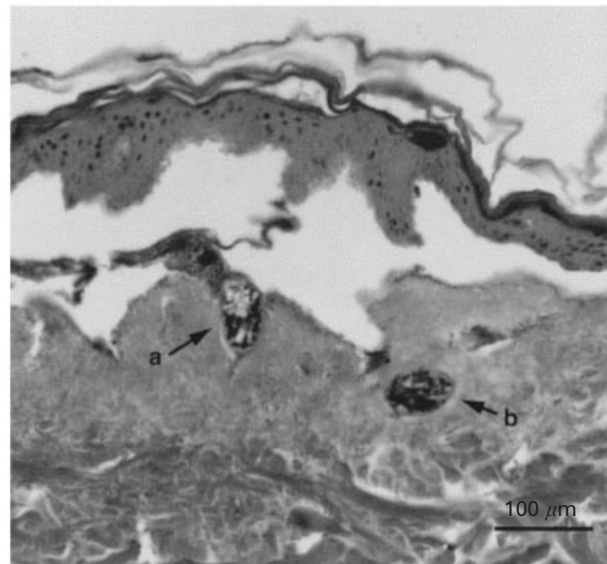

Fig. 4. Schistosomulum penetration after 24 h. H & E stained section showing 2 schistosomula. One (a) has begun to penetrate the upper layers of the dermis, the other (b) is completely embedded in dermal tissue.

Histological examination of the skin showed that the parasites were present in large numbers. Most were to be found in the epidermis or in immediately subepidermal locations. Fig. 3 shows 1 schistosomulum (a) that had moved inwards from the stratum corneum and is now within the viable epidermis and another (b) which had completely penetrated the epidermis and now lies on top of the basement membrane of the epidermis, immediately above the dermal layer. Fig. 4 shows 1 parasite (a) just beginning dermis contact and the other (b) already completely embedded in dermal tissue.

In both the assays described above no differences in findings were apparent when thawed, previously frozen, skin was used instead of fresh skin.

DISCUSSION

The main aim of this work has been to demonstrate the practicality and usefulness of Franz cell technology in the investigation of the skin phase of the establishment of human schistosomiasis. We suggest that this aim has been fulfilled.

Fig. 2 represents perhaps the most detailed analysis yet available of the temporal dynamics of irreversible attachment of schistosome cercariae to living human skin. It was made possible by the straightforward use of skin from cosmetic surgical sources and Franz cells.

The time-course of attachment and presumably of concurrent early penetration events deduced from these experiments allows comparisons with data from other investigations using more indirect tech-

niques. Those of Fusco *et al.* (1993), for instance, using artificial skin were clearly describing an attachment and early penetration process that was dramatically slower than that evident in Franz cell experiments. They saw no irreversible attachment/penetration at 15 min and a maximal rate at 20 h after exposure. In our experiments, by 15 min the attachment/early penetration was already complete and very significant irreversible attachment was achieved after 1 min. Our findings in terms of temporal dynamics are more comparable with those obtained by other workers using laboratory rodents. Wheater & Wilson (1979), for instance, showed that *S. mansoni* cercariae, successfully entering the skin of laboratory mice, reached the base of the epidermis within 30 min.

Perhaps unsurprisingly, the time-course described with Franz cells bears direct comparison with that monitored in a different manner by Stirewalt (1957) using human skin. For 21 cercariae individually observed penetrating human skin, the range of times to achieve superficial penetration of the stratum corneum (and thus not be removable by washing) was 3·1–17·0 min. Behaviour with this time-course would generate temporal dynamics at the population level very like that seen in Fig. 2. In these comparisons of Franz cell findings with those of other workers it should be noted that the control experiments have demonstrated that there is a fixed fraction of cercariae that cannot be recovered (approximately 24 % in these experiments) from the upper cell. This fraction has been ignored in the comparisons that have been made.

Using Franz cells, less than 0·1 % of the cercariae applied to the skin surface emerged as schistosomula into the culture medium of the receptor well within 72 h. This low level of transdermal translocation is perhaps unsurprising for 2 reasons. Firstly, there is much evidence from *in vivo* animal experiments to suggest that the residence times for schistosomula in the skin are considerable. In experiments using hamsters, rats and mice, Miller & Wilson (1978) found that the times to achieve exit from the skin of half of the schistosomular population were 64·5, 70 and 88 h respectively. Almost no exiting had occurred in these species before 48 h. Secondly, and more crucially, there is a consensus from laboratory rodent experiments (Wheater & Wilson, 1979; Wilson & Lawson, 1980; Crabtree & Wilson, 1985) that movement out of the dermis by schistosomula requires them to actively penetrate into the lumen of a venule or lymphatic vessel. They are then translocated from the dermis via the venous circulation or lymphatic drainage. Histological findings in the present study show schistosomula in the dermis. In the Franz cell system, however, neither venules nor lymphatics are perfused, so even if parasites are able to penetrate into their lumina there will be no flow of lumen contents to transport them from the dermis.

The presumption must be that the small numbers of larvae that did reach the receptor wells did so by active penetration through full dermis thickness.

The findings of this study suggest that human skin in Franz cells provides a convenient system for studying, in particular, the early phases of schistosome interactions with human skin. In this way, it can usefully augment the standard approaches which use laboratory rodent models by performing studies directly on viable human skin. It could lend itself well, for instance, to investigation of the efficiency of different topical skin applications which could inhibit cercarial attachment and penetration.

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