Praziquantel-induced tegumental damage *in vitro* is diminished in schistosomes derived from praziquantel-resistant infections

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SUMMARY

The aggressive use of praziquantel to combat schistosomiasis in Egpyt raises concern about the possible emergence of resistance. Eggs from Egyptian patients with praziquantel-resistant infections (not cured by 3 doses of praziquantel) have been used to establish infection-specific schistosome isolates in mice. The response of these worms to the drug was observed *in vitro*, in order to determine if the isolates obtained from these resistant infections were, in fact, less responsive to praziquantel. One of the hallmark effects of praziquantel on schistosomes *in vitro* is a disruption of the worm's outer surface, the tegument. Here, praziquantel-induced tegumental damage is observed in 3 distinct isolates, 2 derived from resistant infections and 1 from an infection cured by a single dose. The isolates from the resistant infections were less susceptible to praziquantel-induced tegumental damage *in vitro*, suggesting that the worms are in some way less responsive to the drug.

Key words: praziquantel, *Schistosoma mansoni*, resistance, tegumental damage.

INTRODUCTION

Schistosoma mansoni infections resistant to praziquantel (PZQ) have been reported among villagers living in the Nile Delta region of Egypt (Ismail *et al*. 1996). These resistant infections were identified when infected patients continued to pass viable parasite ova in their stools after 3 successive doses of PZQ (each dose was given 6–8 weeks apart). From 41 patients with infections not responding to PZQ, ova were isolated and used to establish infection-specific isolates in mice. These infected mice were then treated with various doses of PZQ to determine the dose at which half the mice were cured (the ED_{50}). Seventy-five percent of these isolates had ED_{50} values that were significantly higher than isolates retrieved from patients that were successfully cured after a single dose of PZQ, suggesting that factors attributable to the worm (rather than host variability) were involved in these cases. On the basis of these findings it was concluded that a small percentage (1·5%) of the original sample of infected Egyptian villagers harbored schistosomes that could tolerate high doses of PZQ.

PZQ has 2 well-characterized *in vitro* actions. First is a rapid (within seconds) contraction of the parasite somatic musculature, which can be easily quantified *in vitro* (Pax, Bennett & Fetterer, 1978). It has recently been demonstrated that this *in vitro* action correlates with the drug's *in vivo* action (Ismail *et al*. 1999). In the specific isolates described above, there is a strong correlation between the magnitude of the PZQ-induced contraction response *in vitro* and the efficacy of PZQ *in vivo* or, isolates which produced PZQ-resilient infections in mice proved to have diminished contraction responses *in vitro*.

The second well-characterized *in vitro* action of PZQ is a disruption of the parasite's outer surface, the tegument. The tegumental disruption occurs slower than the drug-induced contraction (within minutes), and is associated with a rapid, drug-induced influx of cations across the tegument (Bricker, Depenbusch & Bennett, 1983). The disruption of the tegument is likely critical to the anti-schistosomal acitvity, since it appears to expose previously secluded parasite antigens, allowing for a more effective immune response from the host. In mice it has been demonstrated that the host immune system can play a vital role in clearing PZQ-damaged schistosomes (Brindley *et al*. 1989).

This paper focuses on the relationship between the efficacy of PZQ *in vivo* and the extent of tegumental disruption induced by PZQ. Specifically, PZQ-induced tegumental damage is compared in 3 different isolates, 2 derived from patients not cured

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by 3 doses of PZQ, and 1 from a patient cured by a single dose of PZQ. The hypothesis is that PZQ-induced tegumental damage observed *in vitro* will be directly related to the *in vivo* efficacy of the drug.

MATERIALS AND METHODS

These studies involved 3 infection-specific isolates of *Schistosoma mansoni* that were maintained in Swiss–Webster mice. Two of these isolates, ER-5 and ER-6, were derived from Egyptian patients who continued to pass viable eggs after receiving 3 doses of PZQ (two 40 mg/kg doses followed by a 60 mg/kg dose). The MO-C isolate was established using eggs obtained prior to treatment from an Egyptian schistosomiasis patient who was cured after a single dose (40 mg/kg) of PZQ. When these isolates were secured in mice and the infected mice were treated with 4 different doses of PZQ, the ED_{50} was 104 mg/kg for the MO-C isolate, 246 mg/kg for the ER-5 isolate and 680 mg/kg for the ER-6 isolate (Ismail *et al*. 1996).

For these studies, the isolates were removed from mice 49–60 days after infection and maintained in RPMI-1640 as previously described (Wolde Mussie *et al*. 1982). Thirty males of each isolate were incubated in RPMI-1640 containing 1·0 μM PZQ at room temperature for 10, 20 and 40 min. In addition, 30 males of each isolate were incubated in RMPI-1640 with no PZQ for 10, 20 and 40 min. Following each period schistosomes were fixed and processed for examination by scanning electron microscopy, as previously described (Bricker *et al*. 1982). A Joel JEM-1200 EXII scanning electron microscope was used to examine the surface of the worms.

RESULTS

Before exposure to PZQ, there were no apparent structural differences between the 3 isolates, and all appeared typical as per previous descriptions (Bricker *et al*. 1983). The major structural components of the dorsal surface of a male worm are the tubercles which protrude from the epidermal surface, which consists of a smooth undulating surface between the tubercles. A unique component of the tubercles are the small spines located around the peak of the tubercles. Before exposure to PZQ, the tubercles and spines all appeared as normal in all 3 isolates, and the isolates were not distinguishable from one another (compare Fig. 1A and B).

When the MO-C isolate was exposed to 1 μM PZQ, disruption of the dorsal surface was evident after 10 min, and by 40 min the typical structural features were almost completely degenerated. After only 10 min of exposure to PZQ, in every MO-C worm examined the dorsal surface was distinctly more ruffled, many of the tubercles were either swollen or collapsed, and the spines on the tubercles became less defined and were often missing (Fig. 1C). At 20 min, many of the tubercles appeared swollen and there was evidence of the compression of the surface between the tubercles into rough folds. After a 40 min exposure (Fig. 1E) the tubercles were very distorted or collapsed, the spines were barely distinguishable and the surface between the spines was rough and irregular. Blebbing, the blistering loss of tegumental membranes, was also very prevalent after 40 min.

PZQ-induced tegumental damage in the ER-5 isolate was qualitatively similar to that observed with the MO-C isolate, but there was an observable difference in the temporal progression of the damage. The most dramatic difference was evident after 10 min exposure to PZQ, when in every case there was less damage found on the surface of the ER-5 isolates as compared to the MO-C controls (Table 1). By 40 min, the damage to the ER-5 surface was quite extensive, although still somewhat less than was observed in the MO-C controls. Specifically, even after 40 min, some spines remained intact and the tubercles, although altered, were less often completely collapsed (Table 1).

PZQ-induced tegumental damage in the ER-6 isolate was notably less (Fig. 1 and Table 1). In fact, after 10 min of exposure to PZQ, the dorsal ER-6 surface was difficult to distinguish from a control surface (Fig. 1D). By 20 min, there was some evidence of damage, but well over 95% of the tubercles observed were intact, without signs of swelling or loss of spines. Even at 40 min, the majority of the tubercles remained intact, despite more evidence of damage (Fig. 1F). Specifically, blebbing was observed in every field, and the surface between the tubercles appeared shrunken and withdrawn.

In general, the rank order of damage induced by PZQ on the surface of the different isolates mirrored the rank order of potency of PZQ against murine infections involving the isolates: MO-C > ER-5 > ER-6.

DISCUSSION

Schistosomes that are less susceptible to the *in vivo* effects of PZQ are also less susceptible to the drug's action on the tegument *in vitro*. In fact, in this limited sample there was a correlation between the responsiveness of the worms *in vivo* and the extent of PZQ-induced tegumental damage observed *in vitro*. The most extensive tegumental damage was observed in the MO-C isolates (ED_{50} of murine infections = 104 mg/kg), while less was observed in the ER-5 (ED_{50} = 246 mg/kg) and even less in the ER-6 (ED_{50} = 680 mg/kg) (Ismail *et al*. 1996). It is important that the least susceptible isolate *in vivo* was also the least susceptible to the drug's action on the parasite surface.

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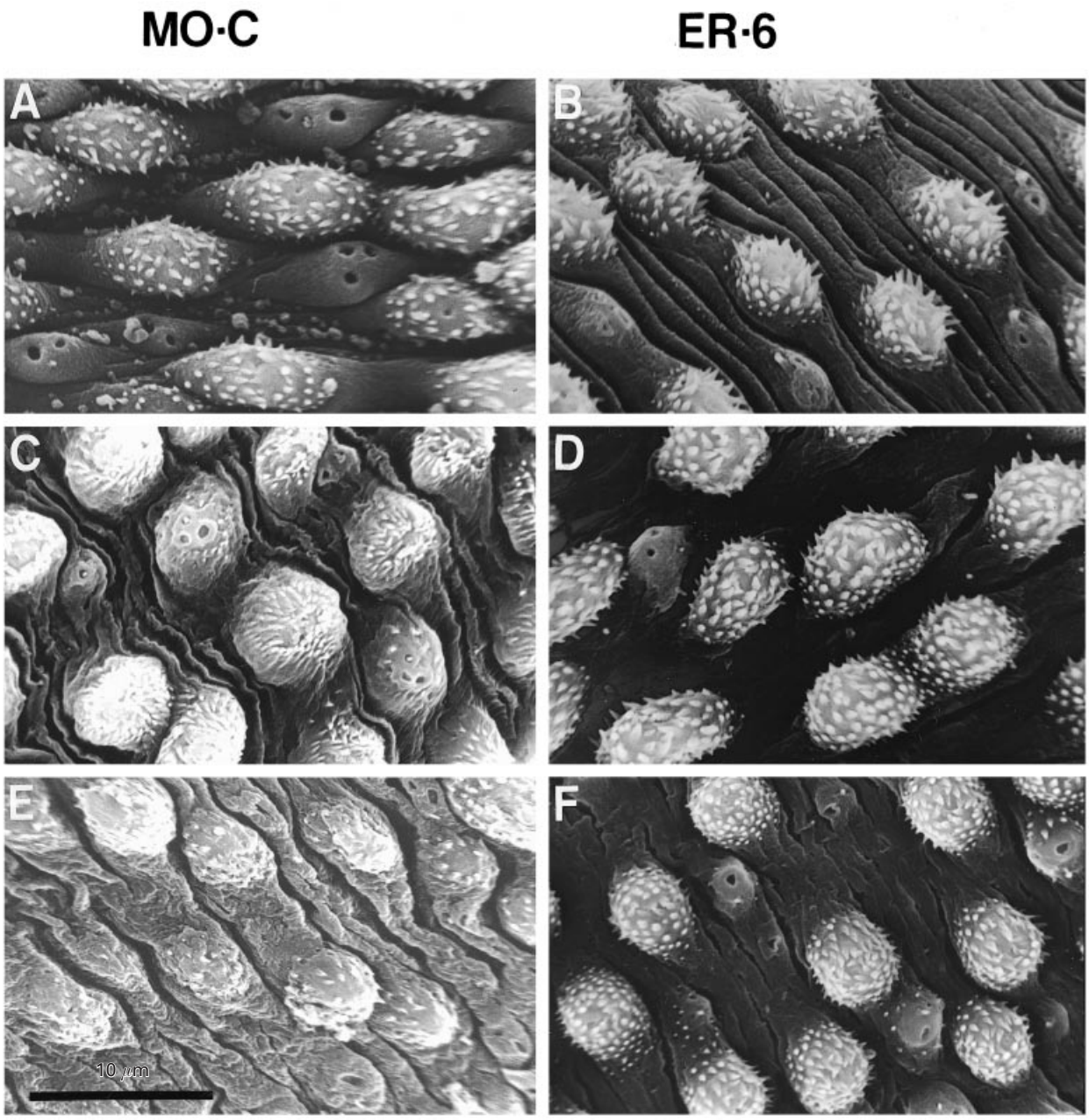

Fig. 1. Photomicrographs of the dorsal surface of male, adult *Schistosoma mansoni* as observed by a scanning electron microscope. These micrographs provide a visual sample of the comparative damage induced by PZQ in the control MO-C isolate (left) and the ER-6 isolate, obtained from a patient not cured by 3 successive doses of PZQ (right).The juxtaposed pictures represent the 2 isolates before exposure to PZQ (A and B), after 10 min of exposure to 1·0 μM PZQ (C and D) and after 40 min of exposure (E and F).

Besides the disruption of the worm tegument, the other readily observable effect of PZQ on schistosomes *in vitro* is a marked contraction of the somatic musculature. It has likewise been demonstrated that worms resistant to the *in vivo* effects of PZQ also had significantly reduced contractile responses to PZQ *in vitro* (Ismail *et al*. 1996). In this case, there was a strong correlation made between the efficacy of PZQ *in vivo* and the contractile response evoked *in vitro*. The diminished responses of these isolates *in vitro* signify that the worms differ from controls in some important way that diminishes their response to PZQ, and these differences contribute to the fact that infections with these worms are difficult to treat with PZQ. There is a clear association between the *in vivo* efficacy of PZQ and these two observable *in vitro* responses.

Reports of schistosomes that are tolerant of high doses of PZQ have appeared in various publications (Fallon & Doenhoff, 1994; Fallon *et al*. 1996; Kusel

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Table 1. Quantification of tegumental damage induced by 1 μM PZQ in three *Schistosoma mansoni* isolates

(These data were obtained by scoring a sample of the photographs produced in the electron microscopy experiments described in the text. The scoring was performed blind, with the scorers having no knowledge of the isolate or the duration of PZQ exposure of each photograph. Each photograph was either scored as demonstrating or not demonstrating the particular type of damage described.)

Duration of PZQ exposure (min)	Percentage of examined fields altered		
	MO-C	ER-5	ER-6
	Disruption of tegument		
0	0 (0/4)	0 (0/4)	25 (1/4)
10	100 (5/5)	25 (1/4)	40 (2/5)
20	100 (5/5)	100 (4/4)	20 (1/5)
40	100 (5/5)	100 (4/4)	80 (4/5)
	Collapse of tubercles		
0	0 (0/4)	0 (0/4)	0 (0/4)
10	80 (4/5)	25 (1/4)	0 (0/5)
20	100 (5/5)	100 (4/4)	0 (0/5)
40	100 (5/5)	100 (4/4)	20 (1/5)
	Loss of spines		
0	0 (0/4)	0 (0/4)	0 (0/4)
10	80 (4/5)	25 (1/4)	20 (1/5)
20	80 (4/5)	50 (2/4)	20 (1/5)
40	100 (5/5)	50 (2/4)	20 (1/5)

& Hagan, 1999). With respect to understanding PZQ's mechanism of action, these schistosomes might offer the opportunity to genetically dissect, through the use of an array of markers specific for the parasite, the gene or genes involved in the drug's action. In addition, PZQ-resistant worms could make it possible to determine whether the observed *in vitro* actions of the drug are related to the phenomena of resistance.

In conclusion, there is good evidence that schistosomes can be isolated from infected patients that are not only resistant to the *in vivo* effects of PZQ (both in original patients and when these isolates are placed in experimental animals), but are equally resistant to the two most prominent *in vitro* effects of the drug. This indicates that the worms themselves are less responsive to PZQ. Although PZQ resistance among schistosomes is not, at this time, significant enough to be considered a public health problem, the fact that dramatically less responsive worms can be isolated from the field demands notice.

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