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ORIGINAL PAPER

Wenshi Wang · Michael Kirschfink · Andreas Ruppel

Schistosoma japonicum and *S. mansoni* cercariae: different effects of protein in medium, of mechanical stress, and of an intact complement system on in vitro transformation to schistosomula

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Abstract The cercariae of *Schistosoma japonicum* were subjected in vitro to treatments known for *Schistosoma mansoni* to generate schistosomula-like organisms. As a technical prerequisite to pipette or to otherwise handle the sticky cercariae of *S. japonicum*, the addition of protein to water or medium was found to abolish the stickiness of cercariae of this species. Shearing forces exerted in vitro by syringe (22 G) passage are known since long to fully transform *S. mansoni* cercariae, but this treatment was found to be much less efficient with *S. japonicum*. Thus, even with very narrow needles (27 G), complete transformation of cercariae was not obtained with *S. japonicum*. Complement, provided by fresh human serum, is also well known to induce rapid transformation of *S. mansoni* cercariae with subsequent killing of the schistosomula. This treatment of *S. japonicum* cercariae induced degeneration of the tails and strongly promoted the transformation to schistosomula-like organisms, but at a much slower pace. These effects were absent from sera either heat-inactivated or depleted of factor B or of complement component C8, but were restored after adding the purified respective complement components. The schistosomula-like organisms of *S. japonicum* were not susceptible to lysis after 1 day of in vitro culture in the presence of 50% fresh human serum, although both cercariae and schistosomula of *S. mansoni* were killed under these conditions. In conclusion, the dynamics of in vitro transformation of *S. japonicum* cercariae differ significantly from those of *S. mansoni,* and complement has a major transformation-promoting activity.

W. Wang · A. Ruppel
Department of Tropical Hygiene and Public Health,
University of Heidelberg,
Im Neuenheimer Feld 324,
69120 Heidelberg, Germany

W. Wang · M. Kirschfink
Institute of Immunology, University of Heidelberg,
Im Neuenheimer Feld 305,
69120 Heidelberg, Germany

A. Ruppel (✉)
Abteilung Tropenhygiene,
Im Neuenheimer Feld 324,
69120 Heidelberg, Germany
e-mail: Andreas.Ruppel@urz.uni-heidelberg.de
Tel.: +49-6221-565044
Fax: +49-6221-565948

Introduction

Cercariae of schistosomes transform into schistosomula during the process of penetrating their host's skin. Transformation involves several steps and morphological as well as physiological changes of the larvae (reviewed by Stirewalt 1974; Stirewalt et al. 1983; Wilson 1987; Bash 1991). Separation of the tails and bodies of cercariae is the most obvious event observed in vitro, but "delayed tail loss" has been reported for *Schistosoma mansoni* in human skin explants (Whitfield et al. 2003). The cercarial bodies subsequently develop into schistosomula and finally grow into adult worms.

Transformation can be induced in vitro to obtain schistosomula. In vitro penetration by cercariae of isolated skin yields schistosomula, which present the characteristics associated with complete transformation, including loss of the cercarial tails and glycocalyx, sensitivity to water, as well as formation of a double surface membrane (Clegg et al. 1972). To facilitate in vitro preparation of schistosomula, techniques for "mechanical transformation" were developed, part of which relied on the application of mechanical stress, e.g., forced passage through a syringe followed by in vitro cultivation of the resulting organisms for several hours (Colley and Wikel 1974). However, even simple incubation of cercariae in medium (Eveland and Morse 1975) or short Vortex treatment results in formation of cercarial bodies with characteristics of schistosomula (Ramalho-Pinto et al. 1974). Several techniques were compared by James and Taylor (1976), Salafsky et al. (1988), and Gold and Flescher (2000). Notably, complement was found to support the transformation process (Eveland and Morse 1975; Greenblatt et al. 1979). All of these early works concern cercariae of *S. mansoni* only.

Cercariae of *Schistosoma japonicum* differ dramatically from those of *S. mansoni* in that they are very sticky. Unlike cercariae of *S. mansoni*, which swim freely in water after release from the snails and can easily be pipetted, cercariae of *S. japonicum* attach to the surface of water, to pipettes, to needles, etc., and very easily form clumps. Consequently, the "ring method" for percutaneous infection of mice (Smithers and Terry 1965), where a suspension of *S. mansoni* cercariae is pipetted within a ring placed on the belly of a mouse, cannot be applied for *S. japonicum*. Rather, cercariae of this species must be applied, due to their stickiness, by the "cover slip method", i.e., by transfer with a needle from the water surface to a water drop on a glass slip (Ruppel et al. 1990). Due to the stickiness of cercariae, we found that the production of "mechanical schistosomula" of *S. japonicum* by published procedures was impossible.

We now solved the technical problem of suspending cercariae of *S. japonicum* in water by the addition of protein. We were then able to transform *S. japonicum* in vitro by the "syringe method" and consequently assess the influence of complement on cercarial transformation. We found that, in many aspects, *S. japonicum* was different from *S. mansoni* and complement promoted transformation.

Materials and methods

Medium for maintenance and culture

Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) was buffered to pH 7.3 with HEPES (0.02 M; Fluka, Buchs, Switzerland) and supplemented with penicillin (100 U/ml; Hoechst AG, Frankfurt, Germany), streptomycin (0.1 mg/ml; ICN Biomedicals, Ohio, USA), and 5% (v/v) heat-inactivated (30 min, 56°C) newborn calf serum (GIBCO).

Sera

Sera were obtained from blood donors (blood bank, Heidelberg) without known histories of parasitic infections. Aliquots were prepared, part of them was heat inactivated (30 min, 56°C), and all sera were stored at −80°C. For use, sera were diluted 1:2 in medium. C8-deficient serum was purchased from Sigma (St. Louis, MO, USA.) and factor B-deficient serum from Calbiochem (La Jolla, CA, USA). Purified C8 (Calbiochem) or purified factor B (Calbiochem) was added at final (physiological) concentrations of, respectively, 160 and 80 μg/ml to reconstitute the respective deficient serum.

Parasites and mechanical treatments

Cercariae of *S. japonicum* were collected from infected *Oncomelania hupensis* snails that were placed in tubes filled with distilled water. These snails were obtained from the Institute of Schistosomiasis Control, Hunan, P.R. China. After the snails were exposed for 12 h to bright light, the cercariae of *S. japonicum*, which normally stick to the water surface, were collected by shaking the top water off into a beaker with medium. In the presence of protein (e.g., newborn calf serum in the medium), cerariae of *S. japonicum* lost most of their stickiness and remained suspended in the medium. Cercariae of *S. mansoni* (a Puerto Rican strain maintained at the Department of Tropical Hygiene, Heidelberg) were obtained from infected *Biomphalaria glabrata* exposed in water to bright light for 1 h. Cercariae of both species were sedimented on ice for 2 h. After the supernatant was removed, one of the following treatments was applied: (1) Cercariae were immediately resuspended (approximately 1,000 cercariae/ml) in warm (25°C) medium and pipetted 20 times up and down with a Pasteur pipette. (2) Warm medium was added and the cercariae were kept on the bench for 15 min to disperse freely (about 1,000 cercariae/ml). The cercarial suspensions were then subjected to shearing forces exerted by passages through a needle, basically as described for *S. mansoni* by Colley and Wikel (1974). Cercariae were sucked into and pushed out of the syringe 20 times by using either 21, 23, or 27 gauge needles. Experiments to compare different needles were performed by the same person (WSW) to ensure all experiments had a consistent force of pressure on the syringe plungers. The parasites were washed in medium (twice with 10 ml) by short, low speed centrifugation. Most of the tails of the cercariae (CT) were removed with the supernatant, and the bodies were further incubated in medium at 37°C for 3 h at room temperature. The resulting organisms were "schistosomula-like". Because it was not the scope of this work to verify that all characters of "true schistosomula" were present, we refer to them, for simplicity, as "cercarial bodies" (CB).

Incubation with sera and assessment of parasites

Cercariae were suspended in medium (1,000/ml) and 100 μl were added per well of 96-well flat-bottom cell culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Normal or complement-deficient sera (see above) were added (100 μl/well; final concentration 50%) with four replicates per experiment. The plates were then incubated at 37°C. After 30 min, 3–4 h, and after 24 h, parasites were observed under an inverted microscope (Olympus, Tokyo, Japan) at 10× magnification, and viable or dead larvae with or without tails were determined. Viability was judged on the basis of morphology, characteristic muscular contractions, and motility. Living CB were actively moving, were translucent, and showed the typical elongated-round shape. Dead CB, in contrast, which had a flattened, rigid, nonmotile appearance, were opaque or granular and often had a protruding ventral sucker. All experiments were repeated at least three times. The results are presented as means±SD.

Results

After being shed from their intermediate host snails, cercariae of *S. japonicum* stick to the water surface and attach to all handling instruments. Preparing cercarial suspensions in water, as done for *S. mansoni*, is impossible with *S. japonicum*. In previous experiments, it had been observed (AR) that the presence of proteins abolished the stickiness of cercariae of *S. japonicum* in water or protein-free media. The addition of bovine serum albumin was found to "release" the stickiness of *S. japonicum* cercariae from the water surface. The addition of albumin or of serum to a final concentration of 5% was sufficient to maintain *S. japonicum* cercariae in suspension. This also allowed pipetting and other handling of cercariae of this species without formation of clumps or aggregates or attachment to glass and plastic surfaces. Although in the presence of protein undisturbed cercariae collected again at the water surface, they could be easily resuspended. Therefore, all following experiments were performed with medium containing 5% of heat-inactivated newborn calf serum.

Transformation of cercariae to schistosomula was performed by exerting various degrees of mechanical stress on the parasites. Starting with cercariae, which had been sedimented on ice, the lowest degree of stress was applied by suspending them in warm medium and pipetting them 20 times with a Pasteur pipette. This method was sufficient to induce tail loss from more than 60% of cercariae of *S. mansoni*, but only less than 20% of those of *S. japonicum*. This pipetting neither kills the cercariae nor damage the resulting tails (CT) or bodies (CB) to any relevant extent (Fig. 1). It should be noted that, with *S. japonicum*, there was always a background of about 10% mortality of CB. This was independent of time and type of experimental treatment. It was an inherent consequence of preparing cercariae from this species, and was not observed with *S. mansoni*.

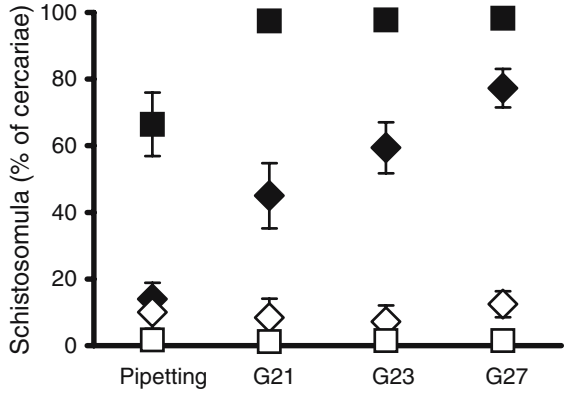

Fig. 1 Mechanical in vitro transformation of cercariae to schistosomula-like organisms by pipetting and passage through needles. Cercariae of *Schistosoma japonicum* (*squares*) or of *S. mansoni* (*diamonds*) were subjected to Pasteur pipetting or to 20 forced passages through needles with different diameters, decreasing from G21 to G27. The resulting schistosomula were counted and classified as living (*solid symbols*) or dead (*open symbols*). Each value represents the mean (±SD) of four to seven independent experiments, each with three replicates. See "Materials and methods" for further details

Increased shearing forces were exerted with 20 passages through needles and starting with a diameter of 0.8 mm (21 gauge). This treatment transformed more than 95% of cercariae from *S. mansoni*, but less than half of those from *S. japonicum*, and the number of dead organisms was negligible (Fig. 1). Further reduction of the diameter of the needle down to 0.4 mm (27 gauge) led to tail loss from up to 90% of the *S. japonicum* cercariae. Under this condition, which exerted the highest mechanical stress, mortality was still virtually absent with CB of *S. mansoni,* but was slightly higher than background level with those of *S. japonicum* [around 15%; (Fig. 1)]. Thus, cercariae of *S. mansoni* readily lost their tails even with low mechanical stress, whereas many of those from *S. japonicum* did so, only when the stress increased.

The influence of the human complement system on transformation of cercariae was tested as representing an "immunological stress". Cercariae were cultivated in the presence or absence of a complete human complement system provided by 50% fresh or heat-inactivated human serum, or by sera deficient in factor B or C8, or reconstituted with these complement components. Results are presented in Table 1. In the absence of complement, i.e., in medium with heat-inactivated human serum, tail loss and transformation was induced within 1 day of culture in part of the cercariae of *S. mansoni*, but not of *S. japonicum*. Viability of cercariae, isolated bodies or tails of cercariae did not decrease to any appreciable extent with either species in the absence of complement. By contrast, in the presence of complement, i.e., of fresh human serum, 40 to 50% of cercariae from *S. japonicum* yielded schistosomula-like CB within 1 day of cultivation. With *S. mansoni*, however, tail loss was dramatically quicker (30 min) than with *S. japonicum* (24 h). In the presence of complement, tail loss was always more pronounced than in its absence. Concomitant with complement-induced transformation of cercariae to schistosomula, complement-mediated toxicity was also observed. Again, *S. mansoni* showed higher susceptibility with virtually all schistosomula being killed after 1 day, whereas for *S. japonicum*, only 20% of CB were dead by this time (Table 1).

The promotion of cercarial in vitro transformation by complement was studied in further detail. Human sera, deficient either in factor B (thus, excluding the alternative pathway of complement activation) or in C8 (thus, excluding the lytic activity of complement), did not induce cercarial transformation, neither early with *S. mansoni* nor late with *S. japonicum*, and there was no difference with respect to observations in plain medium (see above) (Table 1). However, when these sera were reconstituted with the respective purified complement components, i.e., factor B or C8, the relative timing and the degree of cercarial transformation were largely restored for both schistosome species. Similarly, the relative extent of cytotoxicity against the schistosomula was restored, too.

Obvious morphological changes of cercariae were observed under the microscope with cercariae of both

Table 1 Effect of complement and time on the transformation of cercariae from *Schistosoma japonicum* and *S. mansoni* to schistosomula-like organisms (percentage of initial cercariae), and on the survival of larvae

Species	Incubation in	30 min Percentage of LS[a]	4 h Percentage of LS	24 h Percentage of LS (other larvae)[b]
S. japonicum	Medium	0.0±0.0	0.3±0.5	0.3±0.5 (LC)[a]
	iNHS	0.0±0.0	1.8±1.3	1.5±3.0 (LC)
	fNHS	3.8±2.9	8.5±4.0	47.3±8.2 (DC,DS,LC[c])
	Factor B deficient	0.5±0.6	0.5±0.6	1.8±2.1 (LC)
	Factor B reconstituted	2.8±2.1	6.0±2.2	37.3±6.7 (DC,DS,LC[c])
	C8 deficient	1.3±1.9	1.8±1.7	2.5±1.0 (LC)
	C8 reconstituted	9.3±2.5	11.0±2.9	56.5±11.2 (DC,DS.LC[c])
S. mansoni	Medium	0.5±0.6	16.3±4.2	35.8±9.0 (LC)
	iNHS	1.8±2.1	25.8±2.2	59.5±15.2 (LC)
	fNHS	43.0±8.0	0.3±0.5	0.0±0.0 (DS, DC)
	Factor B deficient	0.8±1.0	4.3±2.1	16.3±6.0 (LC)
	Factor B reconstituted	44.8±6.4	1.5±1.3	1.8±1.3 (DS,DC)
	C8 deficient	1.5±1.9	4.8±4.9	9.8±7.5 (LC)
	C8 reconstituted	28.3±4.9	1.5±1.3	0.5±0.6 (DS, DC)

[a]Means of at least 3 independent experiments and standard deviations; *LS* living schistosomula; *DS* dead schistosomula; *LC* living cercariae; *DC* dead cercariae

[b]The organisms listed in brackets are those other than living schistosomula and with which they make up to at least 95% of all organisms (the remaining few percent are not specified)

[c]Among the dead organisms, cercariae and schistosomula are represented in roughly equal amounts; the living cercariae have all degenerated tails and represent 20–30% of all larvae

species placed in contact with human complement, i.e., with fresh human serum or with reconstituted complement-deficient serum. The tails twisted or shortened and, with increasing time, lysed. This was the case with both *S. mansoni* and *S. japonicum* and occurred in parallel with the secretion of the contents from the acetabular glands. However, these complement-dependent processes were much quicker with *S. mansoni* than with *S. japonicum*. Thus, after 30 min in the presence of fresh serum, almost all of the tails from cercariae of *S. manson* had disintegrated (not illustrated), but many cercariae of *S. japonicum* still maintained their normal shape without tail changes and without acetabular secretion (Fig. 2a). Both phenomena took several hours to occur with *S. japonicum* (Fig. 2b–d), and the tails had more or less disintegrated by 1 day (Fig. 2e). Disintegration of tails was not observed in factor B or C8 depleted sera, but addition of these components in a purified form restored tail disintegration (not illustrated).

Discussion

This study shows that in vitro transformation of cercariae of *S. japonicum* differs in several aspects from those of *S. mansoni*. Technically, the stickiness of *S. japonicum* cercariae could be abolished by the simple addition of protein (5% heat-inactivated serum); thus, making them accessible to in vitro handling according to procedures which are standard for *S. mansoni*.

Cercariae of *S. japonicum* were found to be less susceptible than those of *S. mansoni* to mechanical and immunological stress as shown by several approaches.

First, incubation of cercariae at 37°C in medium had no visible effect on *S. japonicum* (Table 1). However, this in vitro condition already triggers partial cercariae-to-schistosomula conversion with *S. mansoni*, as described before (Eveland and Morse 1975; James and Taylor 1976; Greenblatt et al. 1979; Stirewalt et al. 1983) and confirmed in this study.

Second, pipetting through a Pasteur pipette, which represents a relatively moderate mechanical stress, was about fourfold more efficient to transform cercariae of *S. mansoni* compared to those of *S. japonicum*, thus further supporting species differences.

Third, repeated passage through hypodermic needles represents a long-established in vitro-transformation technique for *S. mansoni* (Colley and Wikel 1974). This technique turned out to be much less efficient for *S. japonicum*, and the yield of CB (see Fig. 1) was least with the needle size recommended for *S. mansoni* (22 gauge; Colley and Wikel 1974). Smaller diameters of needles (24 and 27 gauge) enhanced the transformation of *S. japonicum* cercariae. However, the percentage of schistosomula obtained was always below the values for *S. mansoni*. Thus, mechanical stress induces full transformation of cercariae from *S. mansoni* (Gazzinelli et al. 1973; Ramalho-Pinto et al. 1974; Colley and Wikel 1974), but cercariae of *S. japonicum* are relatively resistant to shearing forces. A possible reason could be their smaller size compared to those of *S. mansoni* (333 vs 516 μm; Locker 1983), which may reduce the effect of shearing forces. In addition, the solidity of the body-tail junction could be higher in cercariae of *S. japonicum* than those of *S. mansoni*. To our knowledge, this is the first demonstration of such species-dependent difference in cercarial in vitro transformation.

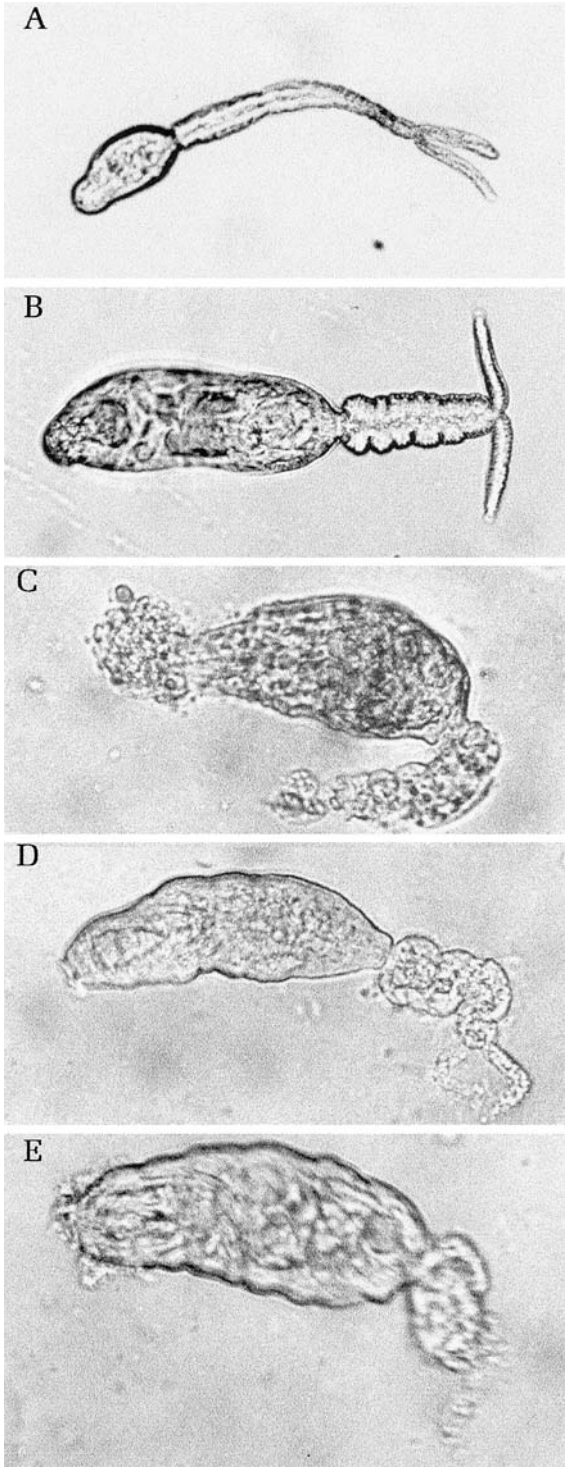

Fig. 2 Influence of complement on cercariae of *Schistosoma japonicum*. Cercariae were incubated with fresh human serum (50%) at 37°C. **a** Zero time and normal morphology, **b** incubation for 30 min with tail shrinking, **c** after 3 h, initial disintegration of the tail and secretion of acetabular contents, **d** further disintegration of tail, **e** close to complete degeneration of tail with a viable schistosomulum-like organism after 1 day of incubation. For best illustration of organisms, various magnifications were used. Tail lysis occurred also with dead cercariae (not illustrated)

For *S. mansoni* cercariae, trauma and disruption were reported to increase when increasingly narrow needles were used (Colley and Wikel 1974), and it was suggested, again for *S. mansoni*, that "schistosomula formed by mechanical techniques may be damaged" although this may not be easily visible (Salafsky et al. 1988). We did not observe a relevant increase in mortality of either species with narrower needles, but cannot exclude that sublethal damage might have been induced with narrow needles. This could be clarified by prolonged in vitro culture or infectivity tests. Nevertheless, the data show the need for protein in the medium to handle, and for narrow needles to transform, cercariae of *S. japonicum* in vitro.

Loss of tails is the most obvious parameter of in vitro-cercarial transformation. Whereas detachment from the CB is the consequence of mechanical transformation, disintegration of the tail was the effect mediated by complement. The fact that the human complement system promotes tail loss was shown for *S. mansoni* by Greenblatt et al. (1979), but the fact that loss was due to degeneration rather than to detachment was not reported. The possible reason was that the tails were already invisible at the time of observation (3 h). In the present work with *S. japonicum*, disintegration was easily visualized during 1 day of culture, including intermediate stages of shrunken or wrinkled tails (see Fig. 2). These observations are proof of a relative resistance against the complement attack of CB, but not of tails. These experiments also demonstrate that the escape from complement-mediated schistosomulicidal activity, which is known since long to occur with *S. mansoni* (Ruppel et al. 1984), does not include cercarial tails and is apparently more pronounced with *S. japonicum*.

The effect of fresh serum on tail loss is, in fact, due to complement, as was shown first by heat-inactivated sera (Eveland and Morse 1975) and then by sera depletion of C3 or one of the terminal components C5 to C8 (Greenblatt et al. 1979). The idea that the alternative pathway of complement was responsible for complement-mediated cytotoxicity against schistosome larvae had been recognized in very early work which, however, concentrated mostly on postcercarial stages (reviewed by Ruppel et al. 1984). The factor B-dependent tail degeneration, observed in this study, supports the role of the alternative pathway of human complement. In this study, the effects on cercariae of *S. japonicum* by an intact complement system as compared to complement-depleted sera were qualitatively compatible with the observations on *S. mansoni*, including those made in earlier reports, but the analogous processes were much slower with *S. japonicum*.

We are aware of only one previous publication dealing with the in vitro effect of fresh human sera on cercariae of *S. japonicum* and comparing this with *S. mansoni* (Yasuraoka et al. 1978). These authors also reported gradual transformation of *S. japonicum* cercariae to schistosomula-like organisms over 1 day of incubation in the presence of fresh human serum. Even though this is compatible with our observations, the results with *S. mansoni* appear as very divergent. After culture of cercariae for 1 day in the absence of serum, these authors

detected no schistosomula for either species, whereas we found about 38% with *S. mansoni* when incubated only in medium with 5% newborn calf serum. On the other hand, they reported 40–50% living schistosomula of *S. mansoni* after incubation of cercariae for 1 day in 50% fresh human serum, whereas this treatment killed all schistosomula in our hands. Although their conclusion "that *S. japonicum* and *S. mansoni* have different optimal requirements for the process of transformation" seemingly agrees with our conclusion, the basis for the statement by these authors is "... the rate and extent of conversion of cercariae to schistosomula in medium with human or rabbit serum was considerably higher in *S. japonicum* than in *S. mansoni*". Our results clearly show for *S. japonicum* that not only the rate (transformed organisms per time interval), but also the extent of conversion are, in contrast, lower for *S. japonicum*. We are unable to explain this contradiction, particularly because the strain of *S. mansoni* used is apparently identical (Puerto Rican origin). In our hands, the prolonged action of complement on *S. japonicum* greatly enhanced transformation, but did not concomitantly kill the larvae within the observation period.

In conclusion, the transformation process from cercariae to schistosomula differs considerably between *S. japonicum* and *S. mansoni*, at least, in vitro. For the infection process, differences between the species are known to exist with respect to the enzymes involved (Ruppel et al. 2004; Chlichlia et al. 2005) and to the speed of migration through the skin (Gui et al. 1995; He et al. 2005; Wang et al. 2005). The phenomenon of delayed tail loss has so far been described only for *S. mansoni* (Whitfield et al. 2003). We hypothesize that this phenomenon might be even more pronounced with *S. japonicum*. We speculate that the apparent in vitro facilitation by complement of *S. japonicum* transformation may lead to a better understanding of early observations (Ruppel et al. 1982) of an apparent complement-mediated increase in the success of infection.

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