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Circulating gut-associated antigens of *Schistosoma mansoni* : biological, immunological, and molecular aspects

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Citation

Dam, G. J. van. (1995, February 9). *Circulating gut-associated antigens of Schistosoma mansoni : biological, immunological, and molecular aspects*. Retrieved from <https://hdl.handle.net/1887/41317>

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Title: Circulating gut-associated antigens of *Schistosoma mansoni* : biological, immunological, and molecular aspects

Issue Date: 1995-02-09

Chapter 11

***Schistosoma mansoni*: *in vitro* and *in vivo* excretion of CAA and CCA by developing schistosomula and adult worms**

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manuscript submitted



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Chapter 11

***Schistosoma mansoni*: *in vitro* and *in vivo* excretion of CAA and CCA by developing schistosomula and adult worms**

Abstract

In this study we describe the excretion patterns of circulating anodic (CAA) and circulating cathodic antigen (CCA) by freshly transformed and developing *Schistosoma mansoni* schistosomula and by adult worms, *in vitro* and *in vivo*. *In vitro*, CAA and CCA were detected in the culture medium of the parasites immediately after transformation, suggesting early excretion by the parasites. During the first days of development more CAA than CCA is excreted, while after about one week the trend is reversed. The excretion of CAA and CCA is influenced neither by addition of red blood cells to the schistosomula culture nor by addition of colchicine. The former observation indicates the absence of an active role of the antigens in digestion, while the latter demonstrates that antigen synthesis and transport to the schistosome gut lumen is not dependent on intracellular microtubules. Culturing adult worms immediately after perfusion of seven-weeks-infected hamsters showed a production of about 20 ng CAA and 100 ng CCA per worm per day and a significantly higher antigen production by the female worms.

In vivo, in mice heavily infected with 1000 *Schistosoma mansoni* cercariae, the antigens were detectable from the third week of infection onwards, with CCA the predominant antigen. After 3½ weeks, the concentration of CCA in serum hardly increased with time, while CAA concentration continued to show a steep increase. After 7 weeks, at the time of perfusion, significantly more CAA than CCA was detected in the serum, but the reverse was true in urine where CCA was the dominant antigen. From the concentrations in serum and urine it was calculated that the worms produce about 50 ng CAA and about 20 ng CCA per adult worm per day. In conclusion, although CAA and CCA levels in serum and urine in general correlate well with worm burden (as determined by egg output), the present study and a literature review show that the actual quantities produced by the worms and detected in the host circulation or excreta may



depend on many factors, such as host and parasite species, clearance rates, or duration and intensity of infection.

Introduction

Since the report of Berggren and Weller (1967) [7], *Schistosoma* circulating gut-associated antigens have been extensively studied by several groups [14–16,29,30,34,43,48,49]. These efforts provided insight in the structure and localization of the antigens and have led to the development of highly sensitive and specific McAb-based ELISAs for the detection of two major gut-associated antigens, circulating anodic antigen (CAA) and circulating cathodic antigen (CCA). These ELISAs are now widely applied for immunodiagnostic and seroepidemiological studies [6,23,26,55,58,59] as well as for studies involving experimental animals [3–5]. Usually, a good correlation of antigen levels with egg excretion is found [6,21,26,40,58], and it is generally assumed that antigen levels in serum and/or in urine reflect the worm burden to a better extent than egg counts [19,27,34,50]. In a number of studies using experimental animals a correlation was demonstrated between CAA and/or CCA levels in serum and worm numbers determined after perfusion [4,5,34,50].

Knowledge about quantitative antigen production by the worms as well as about clearance mechanisms for the circulating antigens in humans is important in order to relate the antigen concentrations to worm burdens before and after drug treatment or in vaccination studies. Until now, clearance of antigens has only been studied in mice. Using McAbs against CAA and CCA, it was shown that injected CAA-anti-CAA McAb immune complexes were cleared at a lower rate in infected animals as compared with uninfected animals, while CCA-anti-CCA McAb immune complexes were cleared equally well [38,39]. Although yet incompletely understood, there are indications from the literature that antigen levels may differ between geographical regions, parasite species and strains, or duration or intensity of infection, even after allowing for levels of egg output [21,23,55]. As it would obviously lead to a better understanding of the underlying mechanisms, we investigated the quantitative excretion patterns of CAA and CCA by freshly transformed and developing *S. mansoni* schistosomula and by adult worms, both *in vitro* and *in vivo*.

Materials and Methods

Parasites and antigens

Adult *Schistosoma mansoni* worms (Puerto Rico strain) were collected from golden hamsters by perfusion of the hepatic portal system with a balanced salt solution, seven weeks after infection with 1500 cercariae. A trichloroacetic acid (TCA)-soluble (7.5% w/v) fraction of homogenized adult worm antigen (AWA-TCA) was prepared as described [29], and used in the ELISA as a reference antigen preparation, shown to contain 2.5% CAA (w/w) and 3% CCA (w/w) as determined using immunopurified antigen preparations [8,57].

For determination of *in vitro* excreted CAA and CCA by adult parasites, seven-week old *S. mansoni* worms were obtained after perfusion of golden hamsters and washed several times with sterile RPMI-1640 medium. Ten male worms, ten female worms, and ten worm-pairs were incubated in duplicate wells of 24-well plates for five days at 37°C in 1 ml RPMI-1640. Worms were still living, motile and paired at day 5 when culture supernatants were taken and stored at -20 °C until tested for CAA and CCA in ELISA (see below).

Preparation and in vitro culture of schistosomula

Schistosoma mansoni cercariae were collected from *Biomphalaria glabrata* previously infected in the laboratory. Schistosomula were obtained by the skin-transformation method and maintained according to the method of [18], as modified by [10]. Schistosomula were fed washed mouse red blood cells on different days to study whether feeding influenced the synthesis and excretion of CAA and CCA. To some of the cultures colchicine was added to a final concentration of 5×10^{-4} M. In control cultures no red blood cells or colchicine were added. The cultures, which contained 200 – 2000 schistosomula, were frequently checked for viability, growth and feeding (if appropriate). At regular intervals samples of the schistosomula culture supernatant fluid were taken and lyophilized. After reconstitution with an equal volume of distilled water CAA and CCA concentrations were determined.

The antigen production per schistosomulum was always determined cumulatively, i.e. the production until the time-point the supernatant was taken. No attempt was made to study whether antigen production is influenced by replacement with fresh medium, as has been done previously [52].

Experimental infection of mice

Two groups of eight week-old male Swiss mice (10 mice per group) were infected with 1000 *S. mansoni* cercariae using the ring method of Smithers and Terry [54]. Blood samples (approximately 150 μ l) were taken from the tails of the mice of the two groups



on alternating days (to avoid severe anemia), starting from day 0 until day 18 resulting in a pooled daily serum sample during the course of infection. From day 18 post infection a blood sample was taken every other day. The mice were perfused on day 45 post infection, worms were counted and serum (by heart puncture) and urine (by bladder puncture) of each individual mouse was collected and stored at -20°C until use.

Antigen detection

The highly sensitive and specific antigen-capture ELISAs for detection of CAA or CCA were performed essentially as described [22,26] with some minor alterations. Among these are the use of a rapid shaking incubator system [42] allowing incubations to be shortened to 15 min and a simplification of the buffer system using just phosphate-buffered saline (35 mM phosphate, 0.15 M NaCl, pH 7.8) with 0.3% (v/v) Tween-20. Briefly, CAA was captured onto McAb 120-1B10-A (IgG1)-coated ELISA-plates (Maxisorp, Nunc, Denmark) and detected using alkaline phosphatase-labeled McAb 120-1B10-A. Color was developed using *p*-nitrophenylphosphate as substrate and absorbance measured at 405 nm. CCA was captured onto McAb 54-5C10-A (IgG3)-coated ELISA-plates and detected using biotin-labeled McAb 8.3C10 (IgM) and streptavidin-alkaline phosphatase conjugate (Dakopatts, Denmark). Serum samples were pre-treated with TCA and urine samples with alkaline carbonate solution (pH 9.6) at 70°C as previously described [20,41]. The relative CAA and CCA concentrations were read against a standard curve of AWA-TCA which contains 2.5% CAA and 3% CCA [8,57]. The detection limit of both assays (for CAA and CCA) was usually around 2 ng AWA-TCA/ml. The antigen levels in serum and urine samples of infected mice were calculated from the standard curve using four-parameter curve fitting and expressed as concentrations CAA or CCA. No adjustment was made for differences in volumes of serum and urine samples (assumed to be due to a variation in the acquisition of samples).

Results

During the first five days of the schistosomula culture the antigen concentrations were low, but well detectable using the sensitive McAb-based ELISAs (Fig. 1). After feeding erythrocytes to the schistosomula the concentrations of both antigens increased, CCA to a larger extent than CAA (Fig. 1). To study this possible influence of feeding, two cultures were set up, one in which schistosomula were normally fed at day 7, and one in which no feeding occurred. At day 11 supernatants were taken from each culture and assayed for CAA and CCA. As shown in Fig. 2, no significant differences between the 2 cultures could be observed. Neither could any influence be observed after adding colchicine, an inhibitor of intracellular transport mechanisms, to the culture before or after feeding at day 9 (Fig. 3).

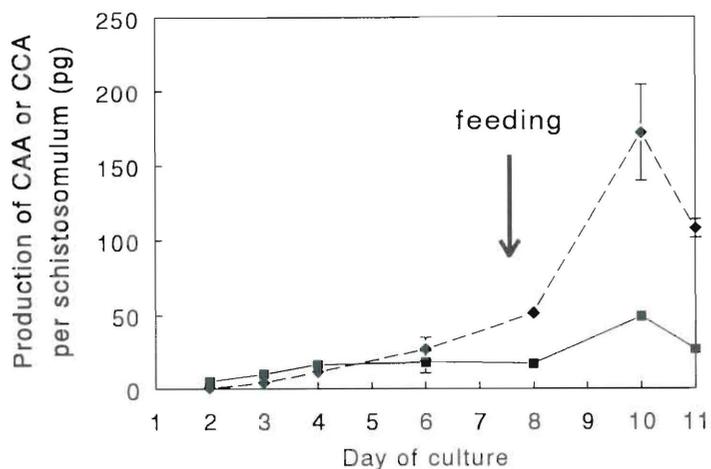


Figure 1. Excretion of CAA and CCA by *in vitro* developing schistosomula. The amount of antigen excreted per schistosomulum is expressed on the different time-points of the culture. Solid lines with squares (—■—) represent CAA and broken lines with diamonds (—◆—) represent CCA (error bars (sometimes too small to be visible) represent standard deviations of two ELISA determinations on supernatants of the same culture).

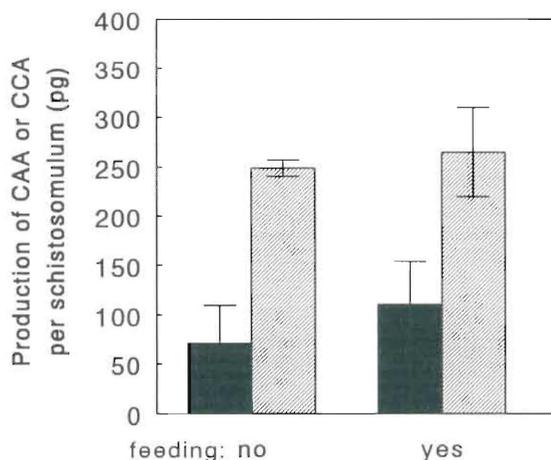


Figure 2. Excretion of CAA and CCA per schistosomulum after 11 days of culture: difference after feeding erythrocytes at day 7. CAA and CCA are represented by closed and hatched bars, respectively (error bars represent standard deviations of two ELISA determinations on supernatants of the same culture).



From Fig. 1 it also appeared that after 5 days of culture the ratio of CAA and CCA concentration inverted. As the amount of antigen produced per schistosomulum was highly variable between different cultures (due to viability and metabolic activity of schistosomula obtained from different batches of cercariae) the development of the ratio's of CAA and CCA concentrations in the culture supernatants with the duration of the culture are depicted in Fig. 4 for two different cultures with and without feeding erythrocytes at day 7. A highly significant difference (Student's t-test, $t=4.31$, $n=41$, $P=0.001$) was observed between the antigen concentration ratio's before and after day 5 for all the culture supernatant samples tested.

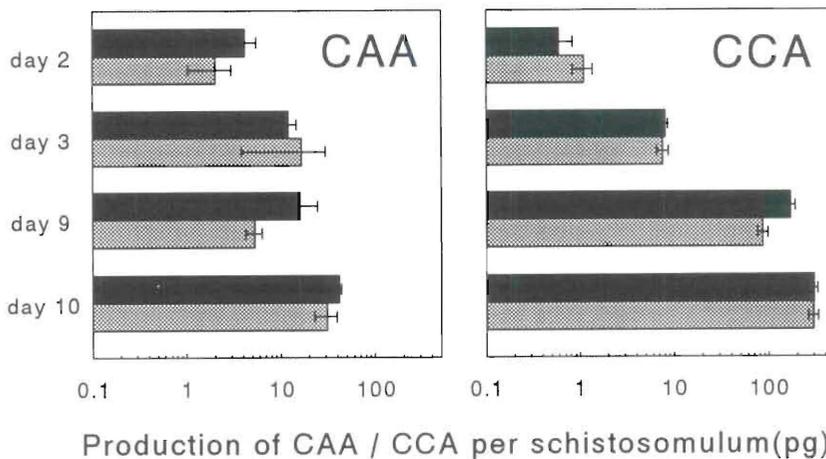


Figure 3. Excretion of CAA and CCA by schistosomula incubated without (closed bars) or with 5×10^{-4} M colchicine (hatched bars) in the culture medium. For the day 2 and day 3 cultures, colchicine was added on day 2 before taking the supernatants, while for the day 9 and 10 cultures, colchicine was added on day 9 simultaneously with addition of the red blood cells and before taking the supernatants (error bars represent standard deviations of two or three antigen determinations performed on the culture supernatant samples).

To compare the antigen production of worms developed in culture with worms developed *in vivo*, a group of mice was infected with 1000 *Schistosoma mansoni* cercariae and the excretion of CAA and CCA was monitored by taking serum samples daily. CCA could be detected from day 16 and CAA from day 18 onwards, while the concentrations of both antigens showed a steep increase thereafter (Fig. 5). Until day 24 more CCA than CAA was detected in the circulation which thereafter was reversed. CAA concentration still increased strongly until the end of the infection period but the CCA concentration rose

only slightly. A small but still significant peak of CAA in serum was observed around day 6.

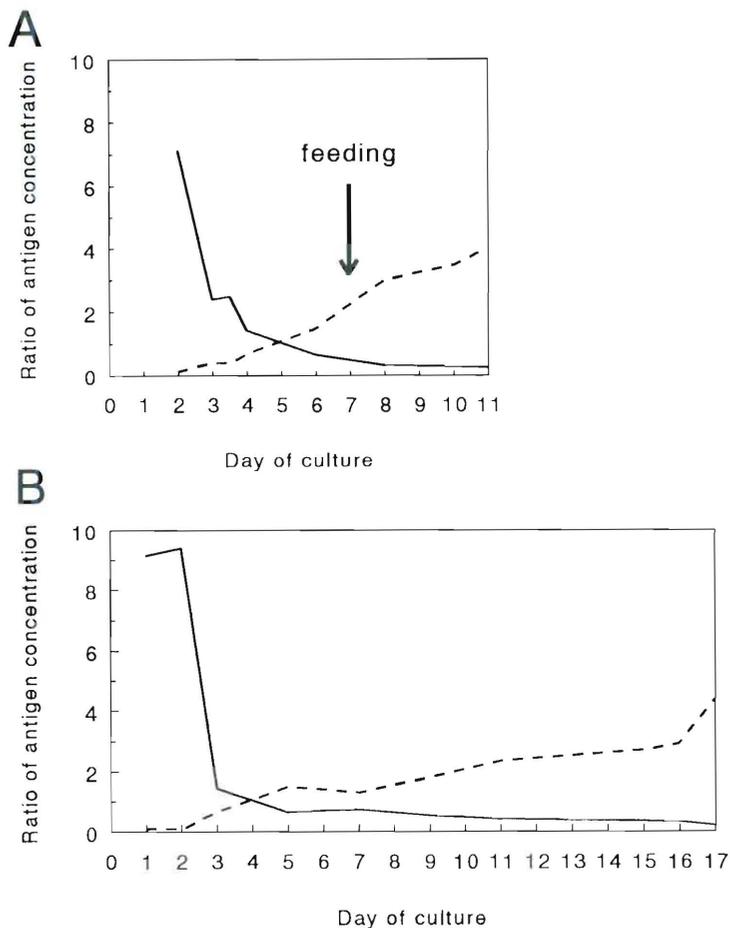


Figure 4. Change in ratio of CAA and CCA in culture supernatants of schistosomula fed at day 7 (A) and not fed at any time (B). Solid lines (—) represent the ratio of CAA over CCA concentration, and broken lines (---) represent the reciprocal ratio.

At day 45 the mice were perfused, worms were counted and serum and urine was collected of each individual mouse, serum usually being about 1 ml, while urine volumes ranged from a few μ l till 1 ml. A comparison of CAA and CCA concentrations in serum and urine showed that the antigen levels in either sample were highly correlated (Table 1). Additionally, the concentration of CAA was very high in the sera but it was less excreted via the urine while the



opposite was true for CCA (Fig. 6). No correlation was observed with total worm counts (Table 1) which only varied between 198 and 491 (male + female) worms per mouse. Many worms were not yet full-grown (probably due to a crowding effect), but the perfusion could not be postponed due to the physical condition of the mice.

Table 1. Correlations between antigen concentrations^a and number of worms in serum and urine of mice 45 days after an infection with 1000 *Schistosoma mansoni* cercariae.

		Serum CCA	Urine CAA	Urine CCA	Number of worms
Serum	CAA	.75* ^b	.83*	.88*	.21
	CCA		.79*	.84*	.22
Urine	CAA			.82*	-.15
	CCA				.04

^a Pearson's product-moment correlation coefficients after log-transformation of the data as in Fig. 6.

^b * = $p < 0.001$

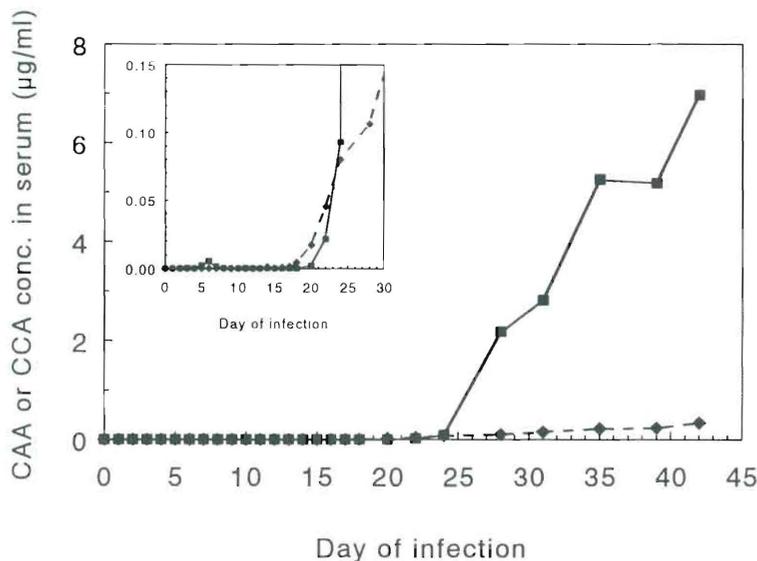


Figure 5. Detection of CAA and CCA in sera of Swiss mice during the development of an infection with 1000 *Schistosoma mansoni* cercariae. Solid lines with squares (—■—) represent CAA and broken lines with diamonds (—◆—) represent CCA. Insert shows the first part of the infection on an extended Y-axis.

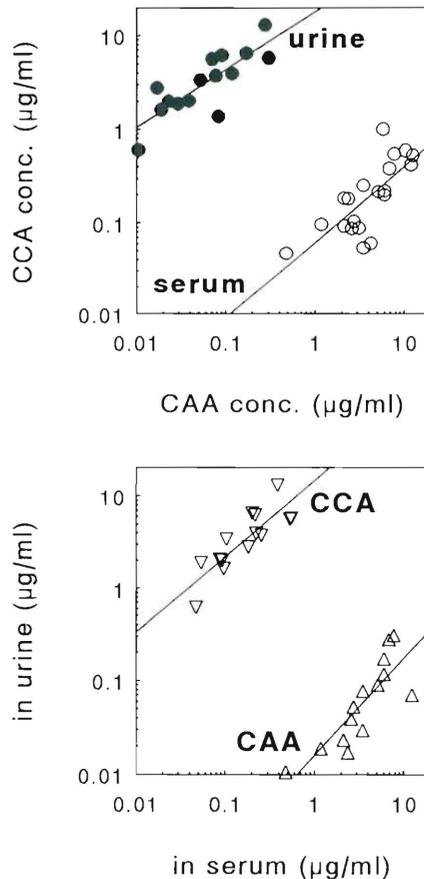


Figure 6. Detection and association of CAA and CCA in serum and urine samples of individual mice at day 45 after infection with 1000 *Schistosoma mansoni* cercariae. Correlation coefficients given in Table 1.

Under the assumptions that (1) a steady-state level of serum antigen concentration was reached (amount of antigen excreted by the worms per day equalled the amount cleared by the host per day), (2) total blood volume of Swiss mice was 3 ml, and (3) urine production per day was about 1 ml, it could be calculated that on average the worms produced 50 ng CAA (range 4 – 118 ng) and 20 ng CCA (range 2 – 37 ng) per worm per day. As the worms were not yet full-grown and the curves for CAA and CCA in Fig. 5 are still rising, these assumptions may not be entirely justified, which would result in higher levels of antigen production per worm.

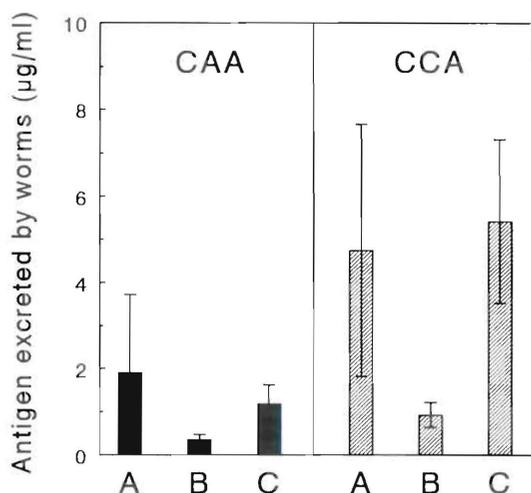


Figure 7. *In vitro* excretion of circulating antigens by 7 week-old adult worms removed from infected hamsters. Worms were cultured for 5 days in 1 ml RPMI-1640 and were still alive and motile when supernatants were taken: A - 10 male and 10 female worms; B - 10 male worms; C - 10 female worms (error bars represent standard deviations of the average antigen concentration of 6 individual culture wells).

To obtain another estimate of the amount of antigen production per adult worm, schistosomes isolated from golden hamsters seven weeks after infection with 1500 cercariae were incubated in RPMI-1640. After 5 days CAA and CCA were determined in the supernatant of the worms all of which were still living and motile (Fig. 7). No difference was observed between the antigen levels detected in the supernatant of the mixed incubation as compared with the culture containing only 10 female worms, while the antigen levels in the culture with only 10 male worms were significantly lower (Fig. 7). This suggests that the major proportion of the antigens is produced by the female worms. After 5 days of culture about 100 ng CAA and 500 ng CCA per female worm was produced ('C' bars in Fig. 7), the male worms producing low amounts as compared with the female worms. Assuming an approximately linear production rate during these 5 days [52], this implies that the female worm produces about 20 ng CAA and 100 ng CCA per day. These values are comparable with the amounts as determined in the infected mice, albeit that *in vitro* more CCA seems to be produced.

Discussion

Although CAA and CCA have been studied extensively in relation to immunodiagnosis of *Schistosoma* infections, few data are available on the production by schistosomula or on the quantitative excretion patterns. As both antigens are gut-associated antigens with comparable ultrastructural localization [24,25], similar results might be expected with respect to the excretion patterns. In the present paper we describe the results of a study which employed *in vitro* cultures of schistosomula or adult worms as well as an experimental, heavy infection of mice with approximately 1000 *Schistosoma mansoni* cercariae.

Directly after transformation CAA and CCA are excreted by the parasites, albeit in very low amounts. A sharp increase of antigen production is observed *in vitro* after day 8 – 10, but *in vivo* the concentrations become detectable only after day 16 for CCA and day 18 for CAA. A summary of literature data on antigen determinations (using different assays) early after infection of various experimental animals with different *Schistosoma* species in Table 2 shows that detectability varies considerably depending on the infection dose and *Schistosoma* species. In humans CAA and CCA can be detected as early as 4 weeks after infection [35,60].

While initially an effect of feeding on the excretion of CAA and CCA by schistosomula was suggested by the observations, further exploration showed that there is a steady increase in antigen production independent of the ingestion of red blood cells. In an 11-day culture no influence could be observed between antigen excretion levels of fed and not-fed schistosomula.

To study the excretion processes in more detail, colchicine was added to the culture media. This drug disrupts intracellular microtubules [11] and it has been reported that addition of colchicine inhibits the ingestion of red blood cells and causes accumulation of secretory granules in the esophageal gland [9]. On the other hand, digestion of hemoglobin in solution is not affected [11]. It was suggested that also the excretion of CAA and CCA occurs via granules or cytoplasmic vesicles [25] but the absence of any effect on CAA and CCA concentrations in culture supernatant indicated that this is probably not the case. De Water *et al.* [24,25] showed that CAA and CCA reactivity was not present in the schistosome esophagus, but only in the Golgi-apparatus, in cytoplasmic vesicles and at the luminal surface coat.

The location of production being in the parasite gut, it might be envisaged that the antigens play a role in digestive processes [43], but regarding the molecular structure of the antigens (polysaccharide side-chains of repeating di- or tri-saccharides, O-linked to a polypeptide chain [8,57]), it is not likely that the



antigens have any enzymatic function. Therefore, the most plausible physiologic function of the antigens is protection of the gut epithelium (like mucins, of which some of the structural characteristics are also shared by CCA [16,57]), rather than involvement in digestive processes.

Table 2. Detectability of circulating antigens in serum of animals experimentally infected with different *Schistosoma* species.

Experimental animal infected with # of cercariae (<i>Schistosoma</i> species)	Antigen detectable from week #	Antigen	Type of assay	Reference
mouse				
25	(mansoni)	7	CCA	McAb-based ELISA [4]
25	(japonicum)	2	CAA	McAb-based ELISA [4]
100	(mansoni)	5	CCA	McAb-based ELISA [4]
100	(mansoni)	5	CAA	McAb-based ELISA [56]
120	(mansoni)	2	CAA	McAb-based ELISA [53] ^a
> 500	(mansoni)	3½	CAA	immuno-electrophoresis with antiserum [7]
600	(mansoni)	4	CAA	McAb-based ELISA ^b
1000	(mansoni)	2½	CAA	McAb-based ELISA this study
1000	(mansoni)	2½	CCA	McAb-based ELISA this study
rabbit				
50	(japonicum)	5	CAA	polyclonal Ab-based ELISA [51]
hamster				
1100	(mansoni)	3	CAA	immunodiffusion with antiserum [34]
1500	(mansoni)	4	CAA	polyclonal Ab-based ELISA [30]
baboons				
1250	(haematobium)	8 ^c	CAA	McAb-based ELISA ^b

^a this study contrasts with others [2,56] and our study with respect to the onset of CAA detectability; it also seems improbable that CAA reaches maximum level already 2 weeks after infection (as shown by Shaker *et al.* (1992) [53]) when the worms are still very small and the gut not yet fully developed.

^b L. van Lieshout, unpublished results

^c tested only at monthly intervals, at 4 weeks borderline positive

From the *in vitro* cultures it was shown that until about day 5–6, more CAA than CCA is excreted. This is already reflected in the antigen content of cercariae as in a preparation of cercarial antigen 60 ng CAA and 7 ng CCA per mg of cercarial antigen was found. The reversal of the ratio CAA/CCA after about day 5–6 occurred irrespectively of the feeding of the cultured schistosomula. In the infected mice, the sensitivity of the assays was not sufficient to detect antigens during the first 2½ weeks of development.

Hereafter, initially more CCA was detected, but this reversed after 24 days post infection, the CAA concentration still increasing strongly until the end of the infection period while the increase of CCA concentration was less pronounced. As clearance influences the antigen concentrations in sera (discussed below), the values might not represent the actual amount of antigen excreted, and conclusions on different production levels for CAA and CCA cannot be drawn. As for the schistosomula, seven-week-old adult worms cultured *in vitro* for 5 days after being perfused from infected hamsters, also excrete more CCA than CAA (Fig. 7), suggesting that from the young schistosomulum stage onwards constantly more CCA than CAA is produced.

Fig. 7 additionally shows that the major part of the antigens is produced by the female worms. It has been documented that female worms are more metabolically active [17,33], resulting in a higher production of gut-associated antigens. The present study confirms these results, showing that the amount of circulating antigens produced by the female worms is about 3–6 times higher than for the male worms. Additional evidence for antigen production predominantly by the female worms is supplied by [56] who found a significant correlation of antigen concentrations in sera of *S. japonicum*-infected mice with female but not with male worm numbers.

Deelder *et al.* [31] calculated a steady state production level of at least 500 ng CAA per worm in heavily infected hamsters. Taking into account that the antigen preparation used for calibration of the assay used by these authors was not completely pure and might consist for less than 10% of CAA [7], their value corresponds well to the values described in the present investigation where for infected mice 50 ng CAA and 20 ng CCA per worm (male + female), and for the *in vitro* cultured adult worms 20 ng CAA and 100 ng CCA per female worm were calculated. The antigen productions per worm determined in sera of mice 10 weeks after infection with 100 *S. mansoni* cercariae, as derived from [56] are considerably lower, namely averages of 3 ng CAA and 10 ng CCA per worm. Differences in clearance rates, mouse and parasite strains, and / or intensity of infection might account for these variabilities in antigen production levels. Studies of CAA production by different *Schistosoma* species in mice are in progress [2].

After 24 days post infection more CAA than CCA is detected in the circulation of infected mice, resulting in about 20 times more CAA than CCA at the time of perfusion. In the urine however, about 60 times more CCA is detected than CAA, and even if the amounts of antigens in serum and urine (assuming a total blood volume of 3 ml per mouse, a urine production of 1 ml per mouse per day and a steady state level in serum antigen concentration) are taken together, three times more CAA seems to be produced than CCA. The reverse is observed, however, for *in vitro* cultured schistosomula (maximal culture length:



19 days), and for 7-week-old adult worms cultured for 5 days after perfusion. This difference could be explained by a preferential clearance of CCA in mice, probably predominantly via the liver [38,45]. Clearance via the liver is not taken into account in the present calculations, but it might play a very important role as immunofluorescent studies could demonstrate the presence of CAA and CCA in Kupffer cells already 1–2 weeks after infection [28,32].

In contrast with our observations, Van 't Wout *et al.* [56] showed that in female BALB/c mice infected with 100 *Schistosoma mansoni* cercariae more CCA than CAA was detected in the serum, while in serum of mice infected with 25 *S. japonicum* cercariae this was reversed. These and the above mentioned studies indicate that the quantities of circulating antigen detected in the serum of infected hosts are dependent on various factors, like host and parasite species, duration and intensity of infection, or pathological condition [23]. This might also have implications for the interpretation of the results of circulating antigen detection which is now more and more applied for serodiagnosis and as a tool in immunoepidemiological studies.

The observations that CAA is detected predominantly in the serum, while in urine CCA shows the highest concentrations, might be well explained by the characteristics of the kidney filtration mechanism. Negatively charged molecules are less well transported than positive or neutral molecules through the basal glomerular membrane, even if they have a similar effective molecular radius [1,12,13].

Although each individual mouse received 1000 cercariae, worm burdens ranged from 200 to 500 worms per mouse. No significant correlation was observed for any antigen concentration with worm burden, which was also found by [30,31], while a positive correlation over a larger range of worm numbers per animal was observed by [5,36,37,50]. It should be noted that the design of the present investigation was not optimal for studying correlations of antigen levels with worm burdens as only one cercarial infection dose was given resulting in a low range in worm numbers (only 2½-fold). In the studies referred to above, these ranges were generally much longer, from 10- to 30-fold. CAA and CCA concentrations in serum and urine correlated highly with each other, as also described for humans by Van Lieshout *et al.* [58]. In epidemiological studies it is generally assumed that antigen levels correlate with worm burdens, but further studies on the clearance of CAA and CCA in humans need to be performed, as the antigen level would be a function of multiple factors [46] and highly influenced by clearance mechanisms. It has already been shown that differential clearance of antigens like CAA and CCA, free or in immune complexes, occurs in mice [38,39,44,45,47]. The presence of CAA-containing immune complexes in mice and hamsters experimentally infected with *S. mansoni* was shown by Deelder *et al.* [31]. However, this study also showed that the production of

CAA-specific antibodies varied between the animals, resulting in a variation in free CAA-concentration and possibly also in different antigen clearance rates [31].

As a conclusion, the present study in combination with a literature review suggests that the amount of antigen detected in the circulation or in the urine of infected mice not necessarily reflects the actual production by the worms, but is influenced by the host metabolic activities which might be different for CAA or for CCA. A statistical association with the worm burden has often been found, but directly relating antigen concentrations to numbers of worms in the host circulation should be performed with great caution.

Acknowledgements

This study is financially supported by a grant from the Netherlands Foundation for Biological Research (NWO/BION) as well as by the Commission of the European Communities under the programme for Science and Technology for Development. We would like to express our thanks to Drs. L. van Lieshout and N. de Jonge for helpful discussions and critically reading the manuscript and to D. Kornelis and T.M. Falcão Ferreira for excellent technical assistance.

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