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

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## Chapter 9

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***Schistosoma mansoni* excretory antigen CCA  
shares carbohydrate epitopes with human  
granulocytes and evokes host antibodies  
mediating complement–dependent lysis of  
granulocytes**

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## Chapter 9

### ***Schistosoma mansoni* excretory antigen CCA shares carbohydrate epitopes with human granulocytes and evokes host antibodies mediating complement–dependent lysis of granulocytes**

#### ***Abstract***

Parasitic worms of the genus *Schistosoma* excrete relatively large amounts of immunogenic glycoproteins (circulating cathodic antigen, CCA) containing polysaccharide side–chains with the trisaccharide Lewis x as a repeating unit. These carbohydrates evoke high titres of specific IgM antibodies which might cross–react with the repeating Lewis x units on the surface of granulocytes. Consequently, this might lead, in the presence of complement to lysis of the granulocytes. In the present study these hypotheses were investigated using anti–CCA mouse monoclonal antibodies (McAbs) and polyclonal antibodies purified from sera from infected humans. By flow cytometry it was demonstrated that the mouse McAbs directed against CCA strongly recognized the granulocytes. It could also be shown that these McAbs as well as anti–CCA IgM antibodies purified from infected human sera caused lysis of granulocytes in a complement–dependent cytotoxicity assay. When compared with sera from healthy controls and with sera from patients with other helminth infections, complement–dependent granulocytotoxicity occurred significantly more in sera from schistosomiasis patients. These *in vitro* observed phenomena may explain the mild to moderate neutropenia occurring in schistosomiasis patients.

#### ***Introduction***

Trematodes of the species *Schistosoma* are blood–dwelling parasites, able to modulate and evade the host immune system in a variety of ways (reviewed in e.g. [5]), like camouflage by acquisition of host antigens [9,20,32], reduction of



surface antigenicity by tegument antigen shedding [15,29] and eliciting parasite-protective blocking antibodies [4,16,22]. Recently, we reported the purification and structural analysis of one of the major antigens associated with the gut of adult *Schistosoma mansoni* worms, the circulating cathodic antigen (CCA) [39]. The antigenic moiety of CCA was characterized as a polysaccharide with the Lewis-x ( $Le^x$ ) trisaccharide as a repeating unit.  $Le^x$  structures present in glycolipids and glycoproteins are found on a number of human cell-types (e.g. granulocytes [17,24,35,37], cells of the urogenital tract [31,43], and adenocarcinoma cells [17,33]) as well as on free oligosaccharides in human milk and urine [21]. Circulating human granulocytes are enriched in  $Le^x$  and carry in relatively high abundance branched N-linked polysaccharides having  $Le^x$  repeating units [35]. Deelder *et al.* [14] demonstrated that the predominant IgM response against *S. mansoni* gut-associated antigens in humans was directed against CCA, as measured in an immunofluorescence assay. In addition, a mild to moderate neutropenia in chronic schistosomiasis patients was observed [3], which might be caused by an inhibitory factor in the sera of these patients delaying the maturation of neutrophils in the bone marrow and spleen [3,30]. Combining these three observations we proposed the hypothesis that excretion of CCA evokes high titres of anti-poly- $Le^x$  antibodies, which are also directed against identical host carbohydrate structures on e.g. neutrophils, thereby causing complement mediated antibody-dependent lysis of these cells. In this context, Ko *et al.* [23] found that a murine protective IgM McAb, raised against *S. mansoni* eggs, recognized the  $Le^x$  determinant (also called SSEA-1, stage-specific embryonic antigen 1), and showed binding to the surface of live schistosomula.

In this study, we have tried to verify our hypothesis that anti-CCA antibodies are involved in granulocyte lysis, by (1) showing that McAbs directed against CCA recognize human granulocytes, which in the presence of complement, cause lysis of the cells, (2) demonstrating that anti-CCA IgM antibodies in sera from schistosomiasis patients correlated significantly with complement dependent lysis of purified human granulocytes, and (3) finally showing that anti-CCA IgM antibodies immunopurified from infected human sera caused lysis of granulocytes in a complement-dependent cytotoxicity assay.

## **Materials and Methods**

### *Production and specificity of McAbs*

McAbs were used from different cell lines obtained from various fusions using immunized mice or mice infected with *Schistosoma mansoni*, *S. haematobium* or *S.*

*japonicum*. Specificity was tested using immunofluorescence and immunoelectrophoresis [12]. McAbs were selected showing reactivity with CCA or another schistosome gut-associated antigen, circulating anodic antigen (CAA) as a control [13]. The primary structure of CAA has also recently been characterized and has been shown to be completely different from the structure of CCA [2], wherefor anti-CAA McAbs were taken as control McAbs.

#### *Human serum samples*

Thirty-nine sera with anti-CCA antibodies were selected for the present study, 29 of which (group A) were used in previous studies on anti-CCA reactivity [14,40], while 10 sera (group B) were randomly taken from sera with positive *Schistosoma* serology (immunofluorescence assay (IFA) on sections of Rossman's fixed adult worms [26]). The sera in group A were from patients whose infections were parasitologically proven by demonstration of *S. mansoni* eggs in the stool. As negative controls, eight sera with confirmed negative *Schistosoma* serology were used (group C). Ten sera (group D) of persons with non-*Schistosoma* helminth infections, exhibiting high antibody titres against *Fasciola hepatica* (n=1), *Onchocerca volvulus* (n=4), *Echinococcus granulosus* (n=1), *Loa loa* (n=2), or *Strongyloides stercoralis* (n=2), were also tested.

#### *Antibody detection assays*

Patient sera were tested in an immunofluorescence assay [26] as well as in an antibody-capture ELISA for anti-CCA IgM antibodies [40]. The cut-off level for the ELISA was determined on the basis of 50 sera from non-endemic persons. The ELISA for anti-CCA IgG antibodies was carried out similarly as described for the IgM assay, with rabbit anti-huIgG (Dako, Denmark) as coating antibodies. As only relative units were relevant in this study results were expressed relative to the serum with the highest reactivity.

#### *Binding of McAbs to granulocytes, using flow cytometry*

Granulocytes from healthy donors were isolated on FICOLL density gradient centrifugation. After 30 min incubation on ice with 100 µg McAb/ml buffer (1% BSA in phosphate-buffered saline, pH 7.6 (BSA-PBS)), cells were washed and incubated for 30 min in the dark with 40 µl FITC-conjugated goat anti-mouse Ig antibodies (1/25 diluted, Becton Dickinson, San Jose, USA). After washing, the granulocytes remained overnight in BSA-PBS at 4°C and the samples were run on a FACStar flow cytometer (Becton Dickinson, San Jose, USA), equipped with an Argon-ion laser tuned at 488 nm, 300 mW. Tenthousand cells were measured per sample.



Three IgM and one IgG3 anti-CCA McAbs were tested for granulocytotoxicity and found to be highly granulocytolytic, while two anti-CAA McAbs (IgM and IgG3) were negative at the same concentrations used (0.1 mg/ml). To evaluate at which antibody concentration McAbs still exhibited granulocyte lysis, one anti-CCA IgM McAb was tested in a dilution series. Lysis still occurred at 0.5  $\mu$ g/ml, while after treatment with DTT no granulocyte lysis could be observed at 30  $\mu$ g/ml. F(ab)<sub>2</sub> fragments of an IgG3 anti-CCA McAb showed no granulocytolytic activity at concentrations (0.5 mg/ml) where the native antibodies were 100% cytolytic, while the anti-CCA reactivity was not diminished as determined in ELISA.

All sera in group A and B and none of the negative control sera in group C were significantly positive in the ELISA for IgM anti-CCA antibodies, confirming results earlier found for this assay [40] (Fig. 2A). In addition, the IgG anti-CCA reactivity was measured in a similar way to compare with the granulocytolytic activity by IgG antibodies (Fig. 2B). Statistical differences of IgM + IgG ELISA reactivities between group A and C and between group B and C were analyzed by Student's t-test:  $t_{A-C}=8.89$ ,  $p<0.0005$ , and  $t_{B-C}=2.97$ ,  $p=0.02$ , respectively.

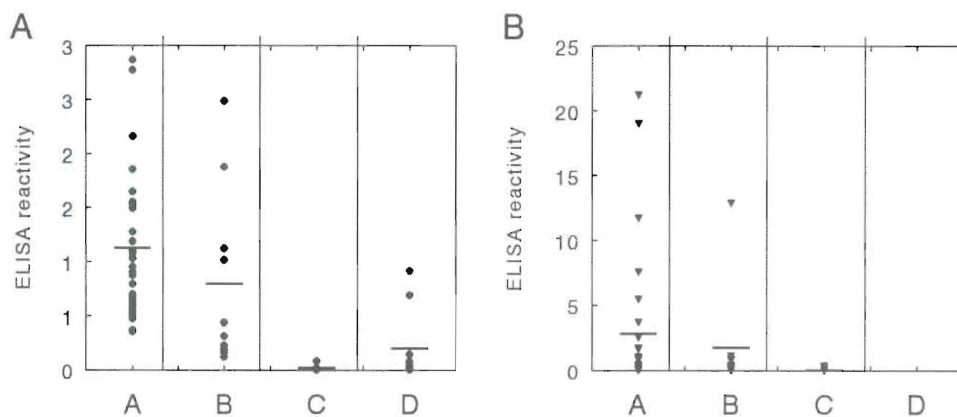


Figure 2. Relative anti-CCA reactivity of IgM (A) and IgG (B) antibodies as determined by ELISA in sera of patients with parasitologically confirmed schistosomiasis (group A), in sera of individuals with only serologically proven schistosomiasis (group B), in sera from non-endemic control individuals or with negative schistosomiasis serology (group C), or by sera from individuals with other parasitic infections (group D). Horizontal bars represent the means of the different groups.

Significant complement dependent lysis of granulocytes caused by IgM and IgG serum antibodies was shown for 83% of the sera of group A and 50% of the



sera of group B. One of the negative control sera in group C was positive, while all sera of individuals with parasitic infections other than *Schistosoma* were negative (Fig. 3A). After treatment with DTT, only 34% of the sera in group A remained positive, whereas all sera in groups B to D were negative (Fig. 3B), indicating that most cytolytic reactivity resulted from serum antibodies of the IgM class. Statistical differences of IgM + IgG antibody-mediated (Fig. 3A) lytic activity between group A and C and between group B and C were analyzed by Student's t-test:  $t_{A-C} = 4.12$ ,  $p = 0.001$ , and  $t_{B-C} = 2.10$ ,  $p = 0.05$ , respectively.

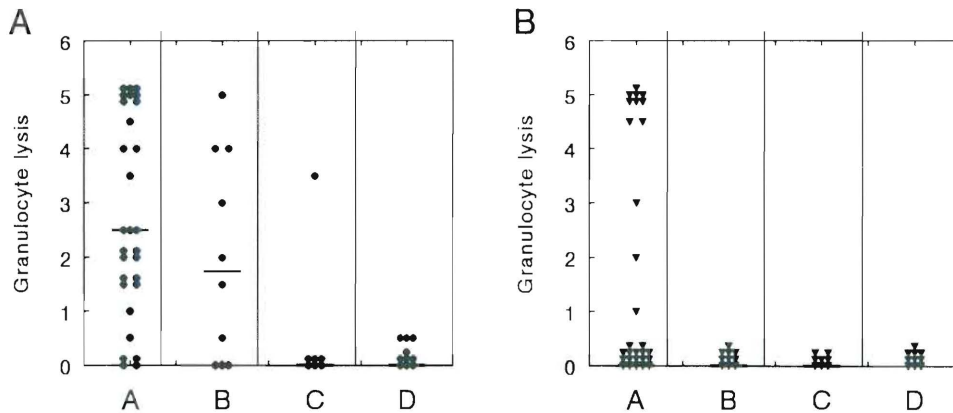


Figure 3. Lysis of granulocytes by sera of patients with parasitologically confirmed schistosomiasis (group A), by sera of individuals with only serologically proven schistosomiasis (group B), by sera from non-endemic individuals or with negative schistosomiasis serology (group C), or by sera from individuals with other parasitic infections (group D). Panel A: untreated sera (lysis by IgM and IgG antibodies); panel B: DTT-treated sera (IgM antibodies are destroyed). Horizontal bars represent the means of the different groups.

If the results for the sera of group A, B and C are combined, the product-moment correlation coefficients for the IgM, respectively the IgG anti-CCA antibody reactivity and the granulocyte-lysis by antibodies of both IgM and IgG class were 0.37 ( $p = 0.01$ ,  $n = 47$ ) and 0.10 ( $p = 0.5$ ,  $n = 47$ ). Scattergrams for these correlations are shown in Fig. 4A and 4B. Inactivation of the complement abrogated the reactivity of two selected sera which were positive using untreated complement.

Three sera of patients with non-*Schistosoma* helminth infections (*O. volvulus*, *E. granulosus*, *L. loa*) showed low to moderate anti-CCA reactivity in the IgM-ELISA (Fig. 2A), but granulocyte lysis was not observed for any of the ten sera (Fig. 3).

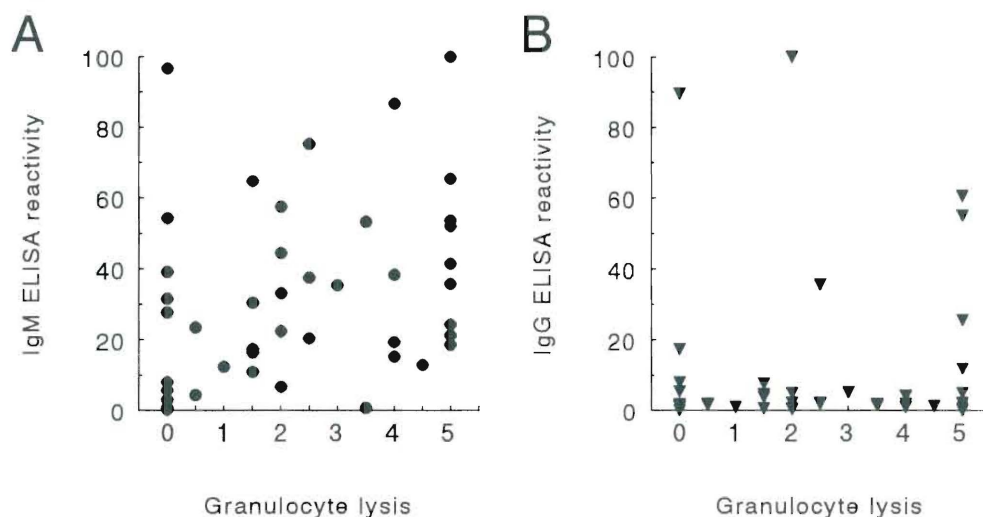


Figure 4. Correlation of granulocyte lysis by human sera with anti-CCA reactivity as measured in ELISA for anti-CCA IgM (A) or anti-CCA IgG (B) antibodies.

After TCA-treatment of lysed granulocytes no reactivity was observed in the CCA-ELISA. However, treatment of 0.3% Tween-20 produced an antigen preparation that was clearly positive, yielding approximately 40 ng CCA-equivalent reactivity per  $10^8$  granulocytes.

No reduction in anti-CCA activity was observed for five schistosomiasis patient sera after absorption with excess granulocytes. However, only one of the two sera which showed granulocytolytic activity before absorption was decreased afterwards. The other serum had a very high IgM anti-CCA reactivity in both the IFA and the ELISA.

Table 1. Reactivity of native sera and immunopurified human anti-CCA antibodies in ELISA and granulocytotoxicity assay.

sample nr.	anti-CCA IgM ELISA		granulocytotoxicity assay		
	native (1/200)	immunopurified (1/20)	native (undiluted) IgM + IgG	IgG	immunopurified (undiluted)
1	0.8	0.6	4	0	-
2	2.8	1.9	5	0	5
3	0.5	0.4	5	2	1
4	1.7	1.0	3	0	2
5	0.8	0.8	5	0	2

Table 1 shows the reactivities in the IgM-ELISA for human anti-CCA antibodies and in the granulocytotoxicity assay of 5 sera, before and after immunopurification on the CCA-coupled Reacti-Gel. Sera incubated with the BSA-coupled gel and eluted by thiocyanate were completely negative in the ELISA.

After preincubation of diluted samples of two highly granulocytotoxic sera with CCA or AWA-TCA at approximately 1 mg/ml a weak to moderate inhibition of cytotoxicity was observed. This indicates that the antigens could inhibit the binding of antibodies in the patient sera to the granulocytes. Similarly, also in the antibody capture IgM-ELISA both anti-CCA McAbs and AWA-TCA inhibited the reactivity in a dose-dependent manner (data not shown).

## **Discussion**

Although schistosome gut-associated antigens CAA and CCA have been extensively studied over the years by several groups [1,6-8,11,12,19,25, 27,28], an experimentally supported function or role of the antigens could not be given. Recent purification and structural analysis of both antigens [2,39] presented new data and tools to extend the previous studies. As a result, it was described that CAA forms complexes with C1q and possibly interferes with the C1q-C1q-receptor interaction [41]. Moreover, CCA was shown to be structural similar to a major granulocyte surface antigen [39], and as high anti-CCA IgM antibody titres are found in schistosomiasis patient sera [14] combined with the mild to moderate neutropenia observed in these patients [3], the hypothesis was developed that CCA is involved in a parasite-induced autoimmunity which causes a neutrophil depletion in the host. In the present paper, we showed evidence supporting this hypothesis by first demonstrating that anti-CCA McAbs bound to a granulocyte surface antigen, which in the presence of complement caused cell-lysis. Additionally, we presented *in vitro* evidence that also predominantly IgM antibodies in schistosomiasis patient sera recognized the granulocytes and induced lysis of the cells by complement. Significant correlations between patient anti-CCA IgM antibodies and granulocyte lysis indicate that the lytic antibodies are indeed directed against CCA. Finally, it could be shown that immunopurified anti-CCA antibodies from schistosomiasis patient sera mediate complement-dependent lysis of granulocytes.

Ko *et al.* [23] found that a mouse McAb which was developed against *Schistosoma* egg antigens and showed *in vitro* antibody-dependent cellular killing of schistosomula, recognized the SSEA-1 determinant (Lewis x, Le<sup>x</sup>). These authors suggested that patient antibodies directed against Le<sup>x</sup> might be a component of concomitant immunity [34]. However, in our hands, infusion of



three anti-CCA McAbs before and during the first days of a *S. mansoni* infection in mice did not result in any reduction of worm burden (unpublished observations). The above authors also suggested the expression of autoimmunity following the induction of anti-Le<sup>x</sup> antibodies, as these antibodies could be directed against circulating granulocytes [23]. In the present study, we have shown that antibodies that are directed against the excretory antigen CCA, are involved in affecting the granulocytes. Since a number of the anti-CCA McAbs recognize egg antigens [13], it is also possible that antibodies induced by egg antigens realize a similar effect.

None of the ten sera from non-*Schistosoma* parasitic infections showed significant granulocytolytic activity, supporting the parasite-specificity of the phenomenon, although anti-CCA activity was detected in three of these sera.

The failure to reduce the anti-CCA reactivity of patient sera by absorption with granulocytes indicates a wider specificity of serum antibodies for CCA than for the recognized granulocyte surface antigen. This is also suggested from the finding that a number of sera having high anti-CCA reactivity were negative against granulocytes. Another explanation for the failure to absorb out the anti-CCA reactivity might be that the number of granulocytes which were used for the absorption was not sufficient, although being theoretically in excess. This is supported by the observation that the serum which was not reduced in granulocytolytic activity possessed also very high anti-CCA reactivity as determined in the IFA or ELISA. A third possible explanation is that only the antibodies with high avidity for CCA account for binding to and subsequent lysis of granulocytes, while in ELISA or in IFA also anti-CCA antibodies of low avidity are detected. This is further supported by the observation that anti-CCA McAbs require multiple Le<sup>x</sup> trisaccharides for binding, as shown by inhibition studies with free Le<sup>x</sup> trisaccharides [39], indicating that the affinity of these McAbs for a single Le<sup>x</sup> is not high enough to inhibit binding of these McAbs to the multiple Le<sup>x</sup>-containing CCA. Stöckl *et al.* [37] similarly found a marked variation in the extent of aggregation induced by different anti-Le<sup>x</sup> McAbs despite similar levels of granulocyte binding.

Evidence that the antigenic structures on granulocytes contain repeating Le<sup>x</sup> epitopes as recognized by anti-CCA antibodies is given by the demonstration that a crude granulocyte antigen preparation reacts positively in the antigen-capture CCA-ELISA. The moderate inhibition of granulocytolytic activity of patient sera by AWA-TCA or immunopurified CCA further supports our hypothesis.

Besides an effect on granulocytes through the induction of anti-Le<sup>x</sup> antibodies, CCA might also have a direct anti-inflammatory and anti-thrombogenic effect by acting as an inhibitor for the adherence of neutrophils to P-selectin on

endothelial cells [18,36]. CCA is detected in the circulation in concentrations sometimes as high as 1  $\mu\text{g/ml}$ , but as an excretory antigen it might locally be present around the parasite in much higher concentrations. It has been described that in mice infected with *S. mansoni* the general neutrophil inflammatory response was decreased, caused by a depletion of mature neutrophils [30]. These authors conclude that this decrease is due to a delay in the maturation of the neutrophils in the bone marrow but the present study shows that it might also be caused by a granulocytolytic process involving anti-Le<sup>x</sup> antibodies and mature granulocytes expressing multiple Le<sup>x</sup> epitopes.

In the present paper we have shown that, for the first time, antibodies induced by a major schistosome antigen are involved in specific lysis of host granulocytes. This confirms the hypothesis that excretion of CCA and subsequent induction of high titres of anti-CCA antibodies might be one of the mechanisms the schistosomes utilize to ineffectuate the host defence system. Although several functions of CCA have been suggested this is the first experimentally supported role of CCA in the host-parasite interaction. However, a direct physiological function of protection of the parasite gut, as supported by the mucin-like nature of the antigen [8,39], might still be the major purpose of the synthesis of this antigen by the parasite. Although schistosomiasis patients show a sensitivity for chronic microbial infections [30], an increased susceptibility of these patients for bacterial infections as compared with patients having other parasitic infections has to our knowledge not been reported. Therefore, it remains to be discussed to which extent this new evasion mechanism of *Schistosoma* is a major or only a minor contribution to the overall capability of the parasite to survive the multiple host immune attacks.

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