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

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Chapter 10

Schistosoma mansoni circulating anodic antigen but not circulating cathodic antigen interacts with complement component C1q

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European Journal of Immunology **1993**; **23**:2807–2812

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Abstract

Adult schistosome parasites, living in the blood vessels of their mammalian hosts, protect themselves against immune damage in a variety of ways. In addition to the tegument, also the intestinal epithelium of the blood-feeding worms is permanently exposed to both the innate and the acquired immune system. In this study, we investigated whether the *Schistosoma* gut-associated antigens CAA and CCA (circulating anodic antigen and circulating cathodic antigen, respectively), which are excreted in relatively large quantities into the host's circulation, might play a role in evading complement attack. Of several complement components tested, only purified C1q showed significant binding to CAA, a negatively charged highly glycosylated glycoprotein. CCA, also highly glycosylated, but neutral or slightly positively charged, did not bind to C1q. CAA bound only to the collagen-like stalks of C1q and not to the globular heads. No detectable interaction of CAA with precursor human C1 was found and CAA did not induce activation of C1 in whole human serum as assessed by consumption of hemolytic C4 activity. Also CAA could not induce activation of precursor C1 *in vitro*. These results suggest that CAA behaves like a receptor for C1q, and might be involved in protecting the vulnerable schistosome gut against complement-mediated attack.

Introduction

Schistosomiasis is characterized by the persistence of adult *Schistosoma* worms in the portal and mesenteric veins of humans and various mammalian species. Although the parasites, living in the blood-stream, are permanently exposed to the host's immune attack, they may be able to persist for up to 40 years in the

host [2]. The schistosome is capable of surviving the significant immune response mounted against it by efficient immune evasion strategies, *e.g.* acquisition of host antigens [6,22], shedding of tegument antigens [30,36], and abundant expression of detoxifying and repair enzymes [26]. While immediately after transformation the schistosomula are highly sensitive to complement-mediated immune damage, they develop an almost complete resistance within a few hours [25]. This is due to a rapid release of complement-activating substances (primarily the glycocalyx) [18] and expression of complement-regulatory proteins on their surface [22,25].

As schistosomes feed on blood, the epithelium of the gut is in principle exposed to all components and cells of the immune system present in the peripheral blood. Lysis of cells by oesophageal gland excretions [23] and acidification of the gut compartment [33] may prevent immune damage. However, the complement system may still exert its function and cause membrane destruction. Antigens originating from the schistosome gut were shown to cause complement consumption in the absence of specific antibody [1,37], although the activation pathway (classical or alternative) could not be identified. Furthermore it has been shown that young schistosomes are able to incorporate decay-accelerating factor [22], a regulator of complement activation both in the classical pathway as well as in the amplification sequence.

In a previous study biochemically purified antigens were used to study interaction with the complement system [37]. However, with regard to the schistosome circulating anodic and cathodic antigen (CAA and CCA), considerable M_r and charge heterogeneity [5,14,28] may have complicated the purification. Since then, McAb specific for CAA and CCA have become available and were used to immunopurify and specifically detect the antigens. The present study was undertaken to determine whether these major gut-associated antigens CAA and CCA, which are excreted into the host's circulation together with undigested food particles, may play a role in regulating complement activation within the schistosome gut or directly around the parasite.

Materials and Methods

Parasites and antigens

Schistosoma mansoni adult worms (Puerto Rico strain) were collected by perfusion of golden hamsters 7 weeks after infection with 1,500 cercariae. Homogenized adult worm antigen (AWA) and a TCA-soluble (7.5% w/v) fraction of AWA (AWA-TCA) were prepared as previously described [13]. CAA and CCA were purified from a 40% ammoniumsulfate supernatant preparation of AWA using Protein A-based immunoaffinity columns prepared as described by Sisson and Castor [34], using mouse McAb

51-4G5-A (IgG3, anti-CAA) and 54-5C10-A (IgG3, anti-CCA) as capture antibody. During the purification procedure, the purity of the antigens was checked by ELISA [10,12] and expressed as percentage of AWA-TCA which was used as reference. On average AWA-TCA comprises 2.5% CAA and 3% CCA, as assessed in several immunopurified preparations.

Buffers

(i) GVB^{++} is isotonic VBS (5 mM diethylbarbiturate, 0.15 M NaCl, pH 7.5), containing 0.1% gelatin, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$; (ii) EDTA-GVB^{++} is 8.6 mM EDTA in GVB^{++}; (iii) GVB^{++}-Tw or EDTA-GVB^{++}-Tw is GVB^{++} or EDTA-GVB^{++} with 0.1% Tween-20; (iv) MgEGTA-GVB^{++}-Tw is GVB^{++}-Tw in which Ca^{++} is chelated using MgEGTA in a final concentration of 5 mM $MgCl_2$ and 10 mM EGTA in *e.g.* GVB^{++}-Tw to study alternative pathway activation; (v) $DGVB^{++}$ consists of one part GVB^{++} and one part $D5W^{++}$, which is 5% dextrose in water (w/v) with 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$.

Purification of complement components

Complement components were purified as already described: precursor C1 [15], C1q, C1q-heads, and C1q-stalks [9], C3 [8], C3b [8], C4 [15], factor B [17], factor H [8], and factor I [16].

ELISA

ELISA with coated schistosome antigens
Standard ELISA methods were used to test binding of schistosome antigens to complement components. Attachment of C1q, C3, C4, or factor H to plates coated with different antigen preparations was detected using horseradish peroxidase (HRP)-conjugated antibody against the individual complement components [9,15]. ELISA-plates (Maxisorp, Nunc, Denmark) were coated with a concentration series of antigen in PBS. After blocking with BSA (0.5% in PBS) the plates were incubated with various solutions as a complement source: to study binding of C1q, 2% normal human serum (NHS) in EDTA-GVB^{++}-Tw or 10 μg/ml purified C1q in GVB^{++}-Tw was used, to study binding of C3 or C4, 0.5% NHS in GVB^{++}-Tw for classical pathway mediated reactions or 5% NHS in MgEGTA-GVB^{++}-Tw for alternative pathway mediated reactions was used, to study binding of factor H, 5% NHS in MgEGTA-GVB^{++}-Tw was used. Complement component specific HRP-conjugates were diluted in GVB^{++}, and color development was performed by adding o-phenylenediamine (stopped by H_2SO_4). Incubation steps were 1 h at 37°C, with the exception of the coating and postcoating which was 15 min at 37°C while shaking [27].

ELISA with coated complement components
As only very weak binding could be demonstrated, the ELISA was reversed: attachment of schistosome antigens to plates coated with purified complement components C1q,

C3b, factors B, H, and I was detected using anti-CAA and anti-CCA McAb. ELISA-plates were coated with the complement components at 5 μg/ml (except factor H which was coated at 2.5 μg/ml) in 0.1 M carbonate buffer, pH 9.6. After blocking with 1% BSA, the plates were incubated with different schistosome antigen preparations in dilution series in VBS at half-ionic strength, and bound antigen was detected using either unconjugated McAb and anti-mouse HRP-conjugates, or biotinylated McAb and HRP-streptavidin. Color development was by 3,3',5,5'-tetramethyl-benzidine and absorbance measured at 630 nm. As a positive control, AWA-TCA was directly coated onto the plates. Incubation steps were 15 min at 37°C while shaking [27].

ELISA with coated C1q fragments

C1q, C1q-heads, C1q-stalks, and BSA as a negative control, were dissolved in PBS at concentrations of 20, 5 and 1.25 μg/ml, and coated onto ELISA-plates. Next, the plates were reacted with 10 μg/ml AWA-TCA in PBS with 0.3% (v/v) Tween-20. After washing, bound antigen was detected using anti-CAA and anti-CCA McAb, similar to the procedure described in the above section. Absorbances were corrected for background using the absorbance of wells which contained buffer only.

Inhibition-ELISA with C1q or collagen type I

In order to study whether the binding of antigen to coated C1q could be inhibited by soluble C1q or collagen type I (a gift of Dr. J.R.O. Hanemaayer), the antigen was mixed with the inhibitors in checkerboard dilution series and incubated on C1q-coated and BSA-postcoated plates. Bound antigen was again detected using McAb and HRP-conjugates.

PEG precipitation of antigen-C1q complexes

The procedure as described by Laclette *et al.* [24] was adapted. Poly-propylene reaction tubes were pre-coated with GVB^{++} to prevent non-specific sticking of C1q. To 50 μl of purified human C1q in $DGVB^{++}$ (containing 0, 2, and 10 μg/ml) an equal volume of the partly purified *Schistosoma mansoni* AWA-TCA (containing both CAA and CCA) in GVB^{++} (containing 0, 4, and 20 μg/ml) was added. This mixture was incubated for 30 min at 37°C with occasional shaking. PEG-6000 (Merck, München, FRG) was added in final concentrations of 0, 1, 3, and 5% (w/v), and the mixtures were incubated for 30 min at 4°C. Tubes were centrifuged for 5 min at 15 000 × g and supernatants collected. The pellets were washed once with 150 μl of a solution with the appropriate PEG concentration in $DGVB^{++}/GVB^{++}$, and finally taken up in 150 μl of GVB^{++}. Supernatants were tested for *Schistosoma* circulating antigens in an antigen capture ELISA routinely used in our laboratory [10,12]. Final concentrations of CAA and CCA were calculated using an AWA-TCA standard curve and assuming that AWA-TCA consists for 2.5% of CAA and for 3% of CCA.

Assessment of binding of C1q and C1 to CAA in a hemolytic assay

Microtiter wells were coated with various concentrations of AWA-TCA, CAA, or BSA for 1 h at room temperature in PBS, and washed. Subsequently 1 μg of C1q (20 hemolytic

units) was added to each well in 100 μl DGVB^{++} and incubated for 1 h at 37°C. After washing the wells twice with DGVB^{++}, 1x10^7 antibody-coated E (EA) containing a dilution of 1/50 of C1q-deficient serum was added to each well and assessed for C1q hemolytic activity. Appropriate reagent blanks and 100% input of EA were included in each experiment. After incubation for 60 min at 37°C the degree of lysis was determined and expressed as units/ml (Z) [15].

Binding of C1 to antigen-coated wells was determined in a similar fashion. In short, after antigen coating, or coating of the wells with aggregated human IgG (AIgG, 1 μg/ml), 10 units of precursor C1 was added to each well in 100 μl DGVB^{++}, incubated for 30 min at 0°C, washed and assessed for bound C1 using EA and C1q-deficient serum.

Activation of C1 by CAA

Activation of C1 by antigen or AIgG was assessed as follows: antigen or AIgG coated wells were incubated with 10 U/ml of precursor C1 at 0°C for 1 h, washed and subsequently incubated with 1 unit of hemolytically active C4 in 100 μl DGVB^{++} for 60 min at 37°C. Thereafter 100 μl EA containing a 1/75 dilution of C4-deficient guinea pig serum was added and residual C4 hemolytic activity was determined.

Results

Specific binding of CAA to C1q

Only very weak binding of the complement components C1q, C3, C4, or factor H to antigen-coated plates was observed with the unpurified or partly purified AWA and AWA-TCA preparations, while plates coated with the affinity-purified CAA and CCA showed no binding. Addition of low concentrations of human serum albumin to increase the coating density of the predominantly carbohydrate antigens had only marginal effect. Indirect coating of the antigens using F(ab')$_2$ fragments of the CAA- and CCA-specific McAb was also ineffective. However, when the ELISA was reversed and the complement proteins (C1q, C3b, factor H, factor I, factor B) were used as coating, significant binding of CAA to C1q was shown (Fig. 1). To inhibit this binding, CAA was mixed with soluble C1q and incubated on C1q-coated plates. As is shown in Fig. 2, binding of CAA was inhibited by C1q-concentrations higher than 0.5 μg/ml. CCA also bound to the C1q-coated plates, but less reproducibly and to a minor extent and could not or only weakly be inhibited by soluble C1q (data not shown). Collagen type I also inhibited binding of CAA to C1q-coated plates in a dose-dependent way but at much higher concentrations as compared with C1q, as shown in Fig. 3.

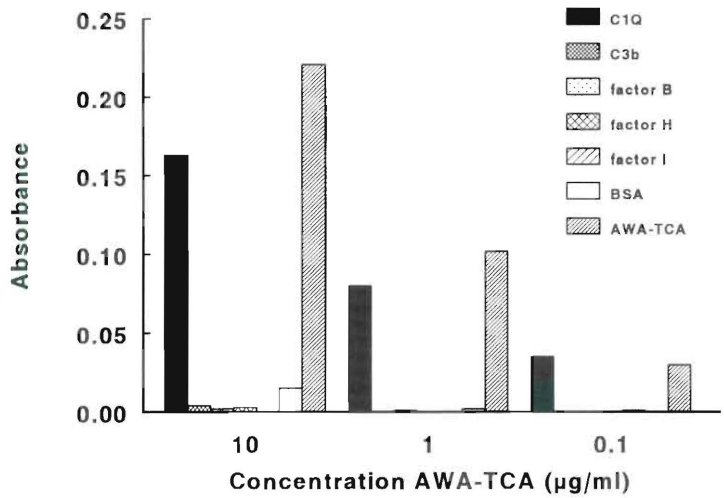

Figure 1. Binding of *Schistosoma mansoni* CAA to complement components in ELISA. Plates were coated with complement components and incubated with increasing concentrations of AWA–TCA, or, as a positive control, coated with AWA–TCA alone. Bound CAA was detected using CAA–specific McAb. Bars represent coatings as given in the figure.

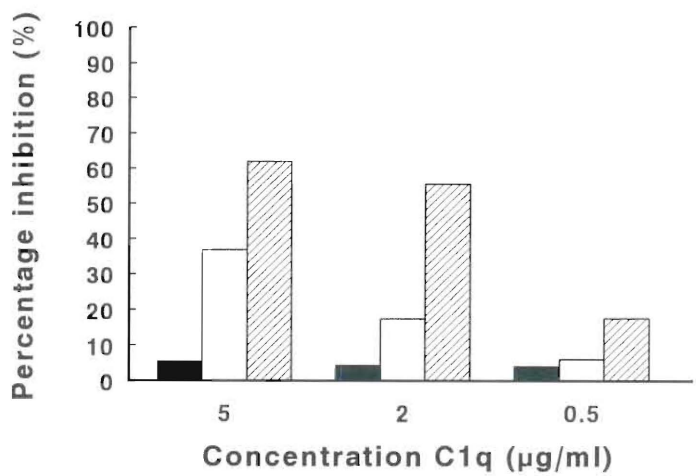

Figure 2. Inhibition by soluble C1q of the binding of CAA to C1q–coated plates. C1q–coated plates were incubated with solutions of C1q mixed with CAA, both in increasing concentrations, after which detection of bound CAA was performed using CAA–specific McAbs. Solid, white and hatched bars represent different concentrations of CAA: 1 µg/ml, 0.1 µg/ml, and 10 ng/ml, respectively.

CAA specifically bound to the collagen–like stalks and not to the globular heads of C1q, as demonstrated in Fig. 4A. CCA–binding was less specific and appeared to be to the C1q–heads only (Fig. 4B).

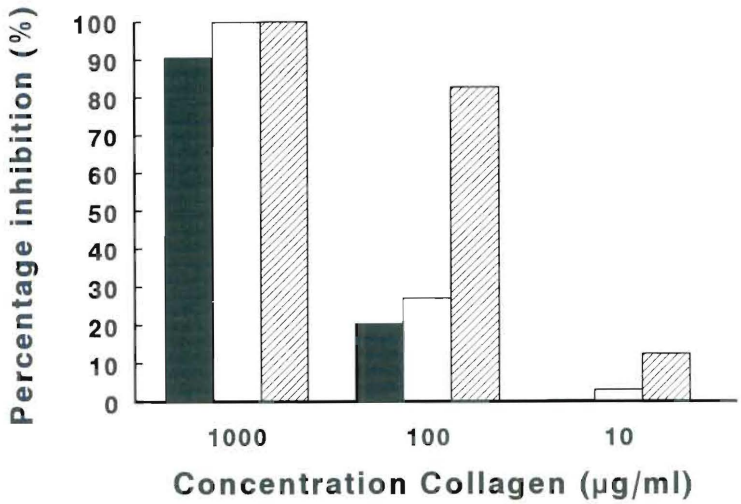

Figure 3. Inhibition by soluble collagen type 1 of the binding of CAA to C1q-coated plates. C1q-coated plates were incubated with solutions of collagen mixed with CAA, both in increasing concentrations, after which detection of bound CAA was performed using CAA-specific McAbs. Solid, white and hatched bars represent different concentrations of CAA: 500 ng/ml, 50 ng/ml, and 5 ng/ml, respectively.

Table 1. Binding of C1 and C1q to *Schistosoma mansoni* AWA-TCA, CAA and controls BSA and AIgG, determined in a C1q hemolytic assay.

Coating	(µg/ml)	C1-bound (Z)	C1q-bound (Z)
AWA-TCA	50	0.17	0.23
	25	0.11	0.14
	12.5	0.02	0.07
CAA	50	0.21	1.30
	25	0.18	0.70
	12.5	0.02	0.40
BSA	50	0.15	0.15
	25	0.07	0.11
	12.5	0.11	0.08
AIgG	50	0.90	1.87

To assess in a more sensitive assay whether CAA also bound precursor C1 and C1q, ELISA wells were coated with AWA-TCA, CAA, BSA or AIgG, washed and interacted with C1q or C1. There was a dose-dependent binding of C1q to CAA while very little binding of precursor C1 was observed (Table 1).

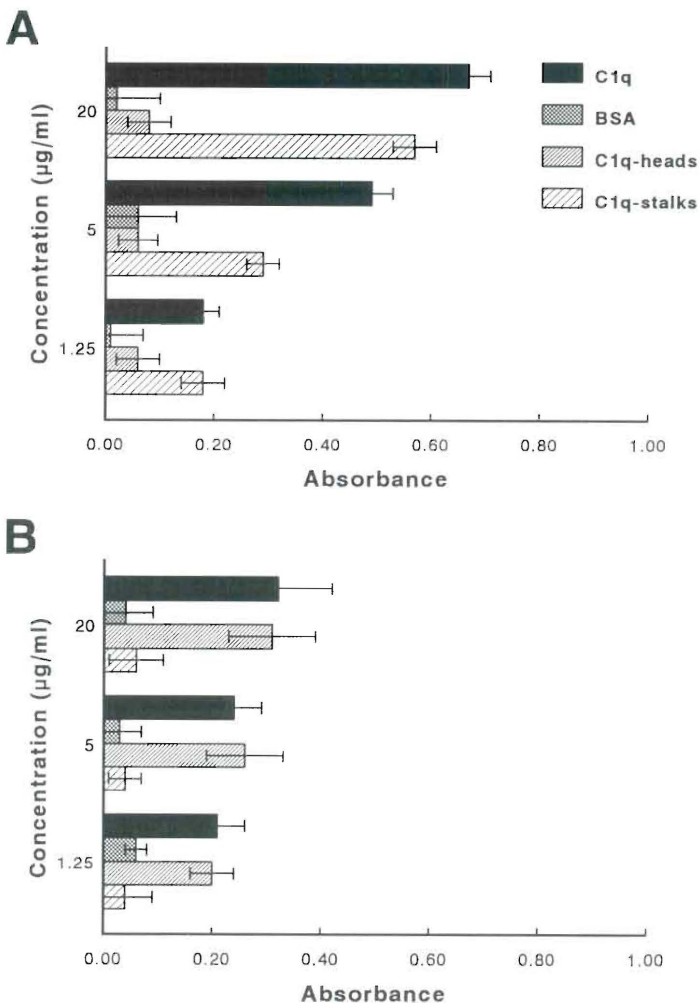

Figure 4. Binding of *Schistosoma mansoni* CAA (A) **and CCA** (B) **to C1q fragments in ELISA.** Plates were coated with C1q, BSA or C1q fragments in different concentrations and incubated with AWA–TCA, which contains both CAA and CCA. Bound schistosome antigens were detected using CAA- and CCA-specific McAb. Bars represent coatings as given in the legend.

Soluble complex formation of CAA and C1q

To demonstrate active formation of soluble complexes of C1q and CAA a binding assay was performed based on the selective precipitation of complexed but not free CAA and C1q by PEG. Precipitation of CAA was increased with increasing concentrations of both C1q and PEG (Fig. 5A). CCA solubility was neither affected by the presence of C1q nor by that of PEG (Fig. 5B).

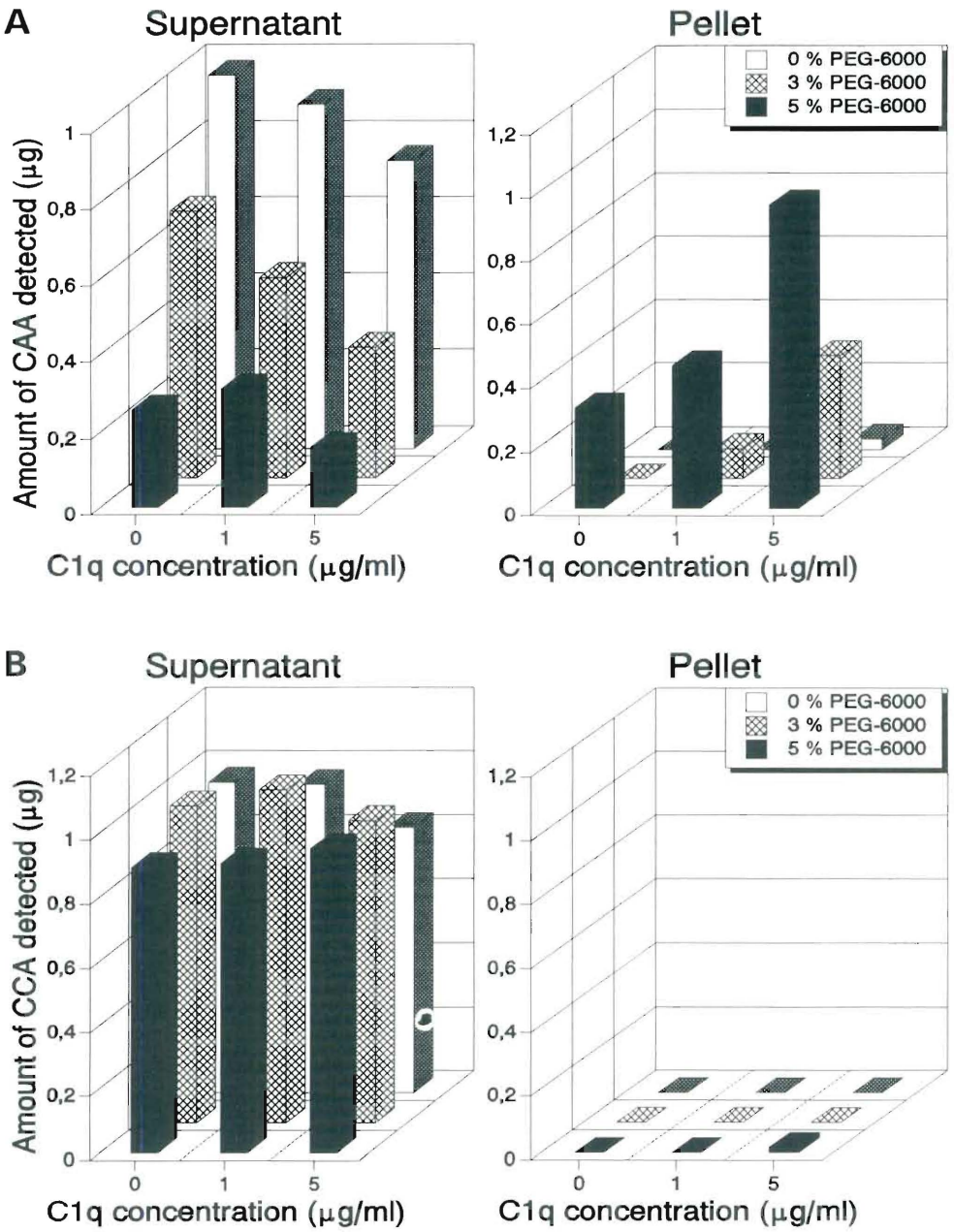

Figure 5. Binding of ***Schistosoma mansoni*** CAA (panel A) and CCA (panel B) to C1q. Soluble complexes were precipitated with increasing concentrations of PEG. White, hatched and solid bars represent 0%, 3%, and 5% PEG–6000 as indicated. Total CAA (A) or CCA (B) was detected by ELISA in PEG supernatants (left panels) and PEG pellets (right panels).

Lack of activation of C1 by CAA

To find out whether CAA was able to activate precursor C1, wells coated with the above mentioned antigens were first incubated with precursor C1, washed and subsequently incubated with C4. While AIgG caused a dose-dependent consumption of C4, neither CAA, AWA-TCA or BSA induced any activation of precursor C1 (Fig. 6).

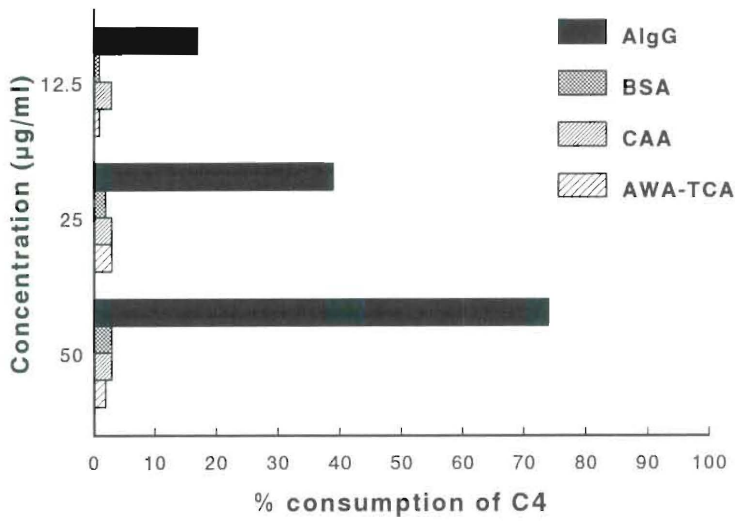

Figure 6. Activation of precursor C1 by *Schistosoma mansoni* antigen preparations. Aggregated human IgG (AIgG) was used as positive control. Bars represent antigens as given in the legend.

Discussion

Activation of complement or binding of complement components by different schistosomal life-stages or antigen preparations has been reported [1,18,24,25,32,37]. Early work of Capron and colleagues who investigated complement consumption by whole worm antigens (AWA) and excretory and secretory antigens (ESA) showed that complement consumption was two times higher for ESA than for AWA when tested in the same concentration [32]. They also demonstrated that ESA directly fixed C1q to a similar extent as AWA and that this C1q binding capacity was predominantly present in the low M_r (<20 kDa) fraction [31]. Van Egmond *et al.* [37] confirmed complement consumption by ESA but they found it to be less efficient than by AWA. These authors also

report complement consumption by different antigen preparations, including the gut-associated antigen CAA (70 Kda) and CCA (40 Kda). Since their study, however, novel and more sensitive techniques have established that the antigen preparations used before were not entirely pure, which may have obscured the results [4,35]. Using McAb to specifically and sensitively detect and purify CAA and CCA we demonstrated soluble complex formation of C1q and CAA, but not of C1q and CCA. However, in contrast to Van Egmond *et al.* [37], the immunopurified CAA and partly purified CCA which was present in AWA-TCA did not show complement consumption in a C4 hemolytic assay. This difference might be due to impurities in the antigen preparations which were used by Van Egmond *et al.* The finding that CAA binds to C1q accords with the observations that polyanions bind strongly to C1q and may activate C1 [7]. In the present investigation, however, binding of CAA to C1q did not lead to activation of C1.

Using purified C1q fragments in ELISA, it is proven that the interaction of C1q with CAA occurs via the collagen-like stalks of C1q. This is confirmed by the inhibition of C1q-CAA complex formation by relatively large amounts of collagen type I. The experiment with C1q fragments also showed that the (variable) binding of CCA to C1q appears to be via the C1q-heads.

Ouassi *et al.* 1981 [29] demonstrated that schistosomula can activate complement through the classical pathway in the presence of IgG, but not through binding of immune complexes. It appeared that IgG peptides resulting from IgG hydrolysis by schistosomula proteases are able to initiate complement activation and cause depletion of C1, C2, and C4. The possibility of a direct action of schistosomula released products on complement depletion was excluded. Indeed, the present study also shows that the excreted products CAA and CCA do not have a direct influence on complement depletion, but that, however, active complex formation of CAA and C1q occurs.

As shown in Fig. 1, CAA bound very efficiently to C1q-coated plates. The lower detection limit as expressed in concentration AWA-TCA (which contains 2.5% CAA) is comparable with the antigen capture ELISA which is routinely operated in our laboratory for detection of circulating CAA. This may imply that the affinity of the binding of CAA to C1q is similar to the (high) affinity of the binding to the McAb that is used in the antigen capture ELISA. It has already been described by Cooper [7] that the affinity of complexes of C1q with polyions may be comparable with the affinity between antigen and antibody in immune complexes.

In several ELISA experiments anti-CAA McAb always clearly showed reactivity, while the positive reaction which anti-CCA McAb detected varied between different assays. This might be due to variability in different preparations of C1q, antigen and McAb. Furthermore, within our laboratory it is a generally observed phenomenon that anti-CCA McAb also cross-react to some extent with CAA (unpublished data). Thus, CAA which bound to coated C1q may, to a minor

extent, also be detected by anti-CCA McAb. However, in Fig. 4 it is shown that CCA, as detected by anti-CCA McAb, bound to intact C1q and to the globular heads of C1q only, while CAA only bound to C1q and C1q-stalks. This indicates that the interaction of CCA with C1q might not only be explained by McAb cross-reactivity. The PEG precipitation experiment showed that no formation of soluble CCA-C1q complexes occurred.

CAA may serve to bind C1q away from the vulnerable schistosome gut endothelium, like O-polysaccharides on the surface of some bacteria (*Salmonella*, *Pseudomonas*) activate the complement distant from the organism's outer membrane [19]. As CAA is an excretory antigen and can be detected in the circulation of the host [10,12] it is theoretically possible that CAA is the causative agent of the markedly depressed complement activity which is observed in serum of infected mice 3 – 6 weeks after infection [3]. On the other hand, chronic schistosomiasis patients do not show a dramatic activation of the complement system [20]. A specific and significant C1q deficiency in schistosomiasis patients has to our knowledge not yet been reported. On the basis of our studies it is not to be expected that the presence of CAA is related to decreased levels of C1q, as the serum C1q concentration (100 μg/ml) is ± 100 times the maximal reported concentration of CAA in the circulation (1 μg/ml, as calculated from the highest CAA titres observed in studies of *e.g.* Deelder *et al.* [12]). Moreover, due to the presence of numerous inflammation reactions around schistosome eggs, increased production of C1q may occur. However, CAA is expressed and released by the microvilli of the syncytium lining the schistosome gut [11], which undoubtedly leads to high local concentrations and would allow binding of all the C1q present.

The observation that CAA binds to C1q via the collagen part but not to precursor C1 suggests that CAA acts as a C1q receptor, and thereby may interfere with the binding of C1q immune complexes with C1q receptor-containing cells such as monocytes, neutrophils and platelets [7]. Following binding of C1q to C1q receptors induces antibody-independent cellular cytotoxicity by various effector cells [21]. Therefore, complement activity as well as complement-mediated cellular activity following binding of C1q to immune complexes present in the schistosome gut may be inhibited after binding of CAA to C1q or C1q-containing complexes.

Active formation of soluble complexes consisting of CAA and C1q was clearly demonstrated (Fig. 5). Detection of C1q simultaneous with detection of CAA and CCA in the PEG supernatants and pellets indicated that C1q precipitation increased with increasing concentrations of AWA-TCA containing the circulating antigens CAA and CCA (data not shown). The relatively low pH present in the schistosome gut (pH = 5 to 6 [33]) does not significantly inhibit the binding of CAA to C1q as was tested in ELISA using buffers of different pH (unpublished data). In summary, we conclude that CAA can form complexes with soluble C1q

which do not lead to complement activation. The interaction of CAA with C1q shows similarities with the C1q receptor–C1q interaction. Therefore it may be hypothesized that CAA plays a role in protecting the vulnerable schistosome gut against complement or complement-mediated attack.

Acknowledgements

This study was financially supported by a grant from the Netherlands Foundation for Biological Research (NWO/BION).

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