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

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Chapter 3

Schistosoma: analysis of monoclonal antibodies reactive with the circulating antigens CAA and CCA

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Chapter 3

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Abstract

Using spleen cells of mice infected or immunized respectively with cercariae or antigen preparations of *Schistosoma mansoni*, *S. haematobium* or *S. japonicum* monoclonal antibodies (McAbs) were produced against the schistosome gut-associated antigens CAA (circulating anodic antigen) and CCA (circulating cathodic antigen). Fusions nearly exclusively produced either anti-CAA (n = 25) or anti-CCA McAbs (n = 55) with a strong isotype restriction (IgM, IgG1 and IgG3) against both antigens, the majority of anti-CAA McAbs being IgG1 and the majority of anti-CCA McAbs being IgM.

The McAbs, which on the basis of their selection were reactive with repeating carbohydrate epitopes of CAA or CCA, were applied in different immunological techniques including immunofluorescence, a dot immunobinding assay and immunoelectrophoresis to study the epitope repertoire. Anti-CAA McAbs were found to be reactive with five different epitopes, none of which occurred as repeating epitopes on eggs. Anti-CCA McAbs, on the other hand, recognized at least ten different epitopes, while 44% of anti-CCA McAbs recognized epitopes common to the adult worm and the egg.

Both CAA- and CCA-epitopes were found to be developmentally expressed at the level of the tegument in cercariae, schistosomula and five-day old lung worms, but in the adult worm were primarily found in the gut. Thus, the production of panels of McAbs has not only resulted in the selection of reagents optimally performing in diagnostic immunoassays, but also allowed a more detailed study of the epitope repertoire of these important schistosome antigens.

Introduction

Since the first publications on the presence of circulating antigens in schistosome infections, and in particular since the description of a circulating anodic antigen [4,25], research in this field has been strongly stimulated by the ultimate goal of developing improved diagnostic assays. Although other circulating antigens were described later [1,26], the two antigens which have been most extensively studied are two glycoconjugates associated with the gut of the adult schistosome: circulating anodic antigen (CAA [19,21]), also known as GASP [37,38], and circulating cathodic antigen (CCA, [19,21]), also known as "antigen M" [8,9]. Initial studies on characterization and serological demonstration of these antigens were based on the use of polyvalent antisera [19,21], while the problems of eliciting specific antisera, particularly against CCA, have stimulated the application of monoclonal antibodies (McAbs). The first of these monoclonals were described in 1983 by our group [17,18] and in 1986 by the group of Capron [39]. Application of these monoclonals had led to a significant increase of our knowledge of the structure of these antigens [5,48], of the clearance of these antigens in experimental animal infection [27], of immunopathological involvement of circulating antigens in experimental animal and in human schistosome infections [14,15,45] and, above all, has expanded the scope of techniques for diagnosis of schistosomiasis.

While initial diagnostic research was only concerned with the mere detectability of circulating antigens in schistosome infections [13,16], subsequent research has addressed follow-up of chemotherapy, reinfection studies, and sero-epidemiological applications not only in *S. mansoni*, but also in *S. haematobium*, *S. japonicum* and *S. intercalatum* [10,11,30,31] infections. At the moment, measurement of circulating antigens is being used to study the kinetics of parasite populations in relation to age (Van Lieshout *et al.*, manuscript in preparation) and to immunity (Agnew *et al.*, manuscript submitted). Most diagnostic studies are based on the application of one or two monoclonals, which often have been described only summarily. During our investigations we have produced large panels of anti-CAA and anti-CCA monoclonals, which were shown to recognize multiple epitopes and which have provided relevant information on the immunochemical reactivity of the two circulating antigens. In view of the rapidly increasing use of these monoclonal reagents, the present study aims at giving a more precise analysis of the available information.

Materials and Methods

Parasites and antigens

S. mansoni (Puerto Rico strain) adult worms were collected by perfusion of golden hamsters 7 weeks after infection with 1500 cercariae. Schistosomula were isolated after mechanical transformation of cercariae, and 5-day old lung worms from lungs of hamsters infected with 5000 cercariae. Adult worm antigen (AWA), and the trichloroacetic acid (TCA)-soluble fraction of AWA (AWA-TCA), soluble egg-antigens (SEA) and SEA-TCA were prepared as previously described [20]. Worm vomitus (WV) was collected by exposing the worms to a 'cold-shock' (0°C) directly after perfusion. The preparation was dialyzed, freeze dried and stored at -20°C (crude WV), or first passed through a 0.45 μm filter to remove insoluble aggregates and then dialyzed and freeze-dried (filtered WV).

At the time of perfusion of infected hamsters, blood and urine was collected by cardiac puncture or bladder puncture, respectively. After treatment with an equal volume of 15% TCA, preparations were centrifuged (25 000 × g for 20 min at 4°C), and the supernates dialyzed overnight against distilled water (4°C), freeze-dried, and designated Hi-TCA (serum) and HiUr-TCA (urine). For experiments in ELISA, CAA and CCA were purified as described by Bergwerff *et al.* [5] and Van Dam *et al.* [48].

Monoclonal antibodies

Hybridomas were prepared from different fusions using different immunization (infection and artificial immunization) protocols, a summary of which is given in Table 1. For all fusions, spleen cells were fused with SP2/0 myeloma cells using polyethylene glycol (PEG). Antibody production was screened with an IFA on male adult worms fixed with Rossman's fixative or on worms present in frozen sections of infected hamster livers. Ascitic fluid was produced by injecting mice with 10^6 hybridoma cells. Ascitic fluid was collected after about 10 days, tested in the IFA for reactivity and stored till use at -20°C. Isotypes of the McAbs were generally determined in IFA using anti-mouse isotype specific FITC-conjugates.

Immunolocalization (IFA and immuno-EM)

The IFA was carried out either on sections of frozen livers of infected hamsters or on sections of adult male worms fixed with Rossman's fixative, or on both [36]. Slides were incubated with McAb (culture supernatant), washed and incubated with an FITC conjugate of rabbit-anti-mouse immunoglobulin antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) diluted 1/50 in PBS (0.035 M phosphate, 0.15 M NaCl, pH 7.8) containing 0.1 mg/ml Evan's Blue. The slides were observed with a Leitz Dialux 20EB fluorescence microscope with the appropriate filter combination for FITC fluorescence. Negative controls were fresh culture medium or ascitic fluid of mice injected with the SP2/0 myeloma-line only. Fluorescence was interpreted visually as

positive or negative (Tables 2 and 3), or on an ordinal scale ranging from 0 to 3, as an average intensity of at least 4 observations at different positions in the sections (Table 4).

CAA and CCA were ultrastructurally localized by electronmicroscopy on 100 nm-thick ultrathin sections of Lowicryl-embedded adult *S. mansoni* worms, using anti-CAA McAb 54-5G10-A labeled with 7 nm colloidal gold and anti-CCA McAb 54-4C2-A labeled with 15 nm colloidal gold according to the procedure described by De Water *et al.* [14].

Immunoelectrophoresis (IE) and Immunodiffusion (ID)

IE was carried out as described by Capron *et al.* [7] on microscope-slides covered with an agarose-gel (1% SeaKem Agarose, FMC Corporation, Rockland, U.S.A.) in 0.08 M Veronalbuffer pH 8.2). AWA (0.8 mg) or AWA-TCA (0.4 mg) was used as antigen, and undiluted ascitic fluid or purified antibody from culture supernatant as antibody. Electrophoresis was performed for 3 hours at 50 V and 3.5 mA per slide. After diffusion, washing and drying, the slides were stained with Amido-Black (Merck, Darmstadt, Germany).

Table 1. Infection and immunization protocols for the various fusion numbers of the hybridomas used in this study.

Fusion nr.	Mouse strain	Primary infection[a]	Primary immunization	Booster	Fusion time (wpi)[b]
5	Balb/c	*S. mansoni*			58
22	Balb/c	*S. mansoni*		*S. mans.* AWA-TCA	46
24	Balb/c		*S. japonicum* CA	*S. jap.* CA	9.5
25	Balb/c		*S. japonicum* CA	*S. jap.* CA	10
27	Balb/c		*S. japonicum* CA	*S. jap.* CA	11.5
51	Swiss	*S. mansoni*			8.5
54	Swiss	*S. mansoni*			8.5
114	Balb/c	*S. mansoni*			8
120	Balb/c		*S. mansoni* AWA	*S. mans.* AWA	26
128	Swiss	*S. mansoni*		*S. mans.* AWA	12
141	Swiss	*S. mansoni*			6
145	Balb/c		*S. mansoni* AWA	*S. mans.* AWA	9
147	Swiss	*S. mansoni*			10
179	Swiss	*S. mansoni*			11
180	Swiss	*S. mansoni*			12
257	Balb/c	*S. haematobium*			8
259	Balb/c	*S. haematobium*			12
273	Balb/c	*S. japonicum*			7
274	Balb/c	*S. japonicum*			7

[a] Except for *S. haematobium* (230 cercariae/mouse) all infections were done with 100-140 cercariae/mouse.

[b] Abbreviations: wpi = weeks *post infection* or *post first immunization*, AWA = Adult worm antigen; AWA-TCA = Trichloroacetic acid-soluble fraction of AWA; CA = Circulating Antigens present in TCA soluble fraction of *S. japonicum* infected rabbit serum [42].

Dot immunobinding assay (DIBA)

In the DIBA, solutions of AWA, AWA-TCA, SEA, SEA-TCA, WV, Hi-TCA and HiUR-TCA in PBS (1 mg/ml) were used as antigens. One μl antigen solution was spotted on strips of nitrocellulose paper (0.45 μm, Schleicher und Schuell, Dassel, Germany), heated to 80°C for 1 h, blocked with BSA, and successively incubated with McAb solutions, peroxidase-conjugate of rabbit-anti-mouse immunoglobulin antibodies (Dakopatts, Glostrup, Denmark) and substrate solution (1.4 mM diaminobenzidine, 1.4 mM 4-chloro-1-naphthol and 4 mM H_2O_2). Intensities of the dots were interpreted visually as negative (-), weakly positive (±) and (strongly) positive (+).

SDS-PAGE and Western-blotting

AWA was electrophoretically separated in the Laemmli system [33]: 5 mg AWA/ml sample buffer (63 mM Tris-HCl pH 6.8, 3.3% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) 2-mercaptoethanol) was heated for 2 min at 100°C and applied to a 12% or 8% polyacrylamide gel. After electrophoresis the separated proteins were blotted [47] at 4°C on PVDF membrane (Millipore Corporation, Bedford, U.S.A.) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol at pH 8.8. After blotting the membrane was cut into strips which were incubated with the McAb solutions diluted in 5% dried skimmed milk in PBS, washed, incubated with the peroxidase-conjugate of rabbit-anti-mouse immunoglobulin in 5% dried milk in PBS, washed again, and finally incubated with the substrate solution as described above for the DIBA. As controls, negative ascitic fluid and McAbs directed against 32 kDa and 36 kDa gut-associated proteinases were included.

Enzyme-linked immunosorbent assays

The antigen-capture ELISAs for CAA and CCA were performed as described [13,16] with the exception that a rapid shaking incubator system was used which allowed the incubations to be shortened to 15 min [35]. Briefly, AWA-TCA or immunopurified CAA and CCA preparations (as described in [5,48]) were captured in various concentrations onto McAb 120-1B10-A (anti-CAA)- or 54-5C10-A (anti-CCA)- coated ELISA-plates (Maxisorp, Nunc, Denmark) and detected using alkaline phosphatase-labeled McAb 120-1B10-A (anti-CAA) or biotin-labeled McAb 8.3C10 (anti-CCA) followed by streptavidin-alkaline phosphatase. Color was developed using *p*-nitrophenylphosphate as a substrate, incubating overnight at 4°C and absorbances were measured at 405 nm.

Data analysis

Reactivity patterns of McAbs in the IFA, IE and DIBA were compared (Tables 2 and 3). Clearly different reactivity patterns of one or more monoclonals were considered to represent recognition of a "unique" epitope.

Results

From 19 fusions with spleen cells of mice which either had been immunized with antigen preparations containing CAA and CCA, or had been infected with *S. mansoni*, *S. japonicum* or *S. haematobium* cercariae, a total of 25 McAbs reactive with CAA and of 55 McAbs reactive with CCA were isolated. Data on the characterization of these antibodies are given in Tables 2 (anti-CAA McAbs) and 3 (anti-CCA McAbs). A number of McAbs showing identical reactivity patterns in all assays in Table 3A were combined under one representative McAb as indicated in Table 3B. It is striking that, with the exception of fusions 51, 54, and 114, only McAbs reactive with one of the two antigens could be isolated from one and the same fusion. In addition, there was a strong isotype restriction against both antigens, in that only IgM, IgG3 and IgG1 isotypes were found. For respectively CAA and CCA these isotypes constituted 28%, 80% (IgM), 12%, 5.5% (IgG3) and 60%, 14.5% (IgG1). Monoclonals were firstly selected on the basis of gut-associated fluorescence in an IFA on sections of adult worms fixed in Rossman's fixative, and secondly on the characteristic reactivity in IE.

Immunolocalization (IFA and immuno-EM)

In the IFA, all anti-CAA antibodies gave a strong fluorescence of the syncytium lining the gut of the male and female worms (Fig. 1b). Characteristic for anti-CAA McAbs was the phenomenon of antigen "detaching" from the syncytium or the intestinal lumen (Fig. 1c), while a number of IgM anti-CAA McAbs (*e.g.* McAb 25-9B10-A), in addition to gut-staining, showed a reactivity with structures, probably nuclei, within the parenchyma (Fig. 1a). In an IFA on livers of *S. mansoni* infected hamsters, anti-CAA McAbs recognized antigen present in Kupffer cells while eggs were negative and only showed autofluorescence (Fig. 1d). The same group of IgM McAbs mentioned above was reactive with miracidia on frozen sections and with nuclei in liver parenchyma on sections fixed in Rossman's fixative.

Anti-CCA McAbs, likewise, always showed a strong reactivity with gut syncytium on sections of adult worms fixed in Rossman's fixative (Fig. 1e). This fluorescence was in general more "defined" than that of anti-CAA McAbs, and not "detached" from the syncytium. On sections of livers of infected hamsters, antigen in Kupffer cells was recognized by all McAbs, while a significant number (29%) of the IgM McAbs reacted strongly with antigen at the level of the shell of eggs present in the liver tissue (Fig. 1f).

In the IFA, anti-CAA and anti-CCA McAbs raised against *S. mansoni*, *S. haematobium* or *S. japonicum* showed a same localization and intensity, regardless of the species used as a source of antigens.

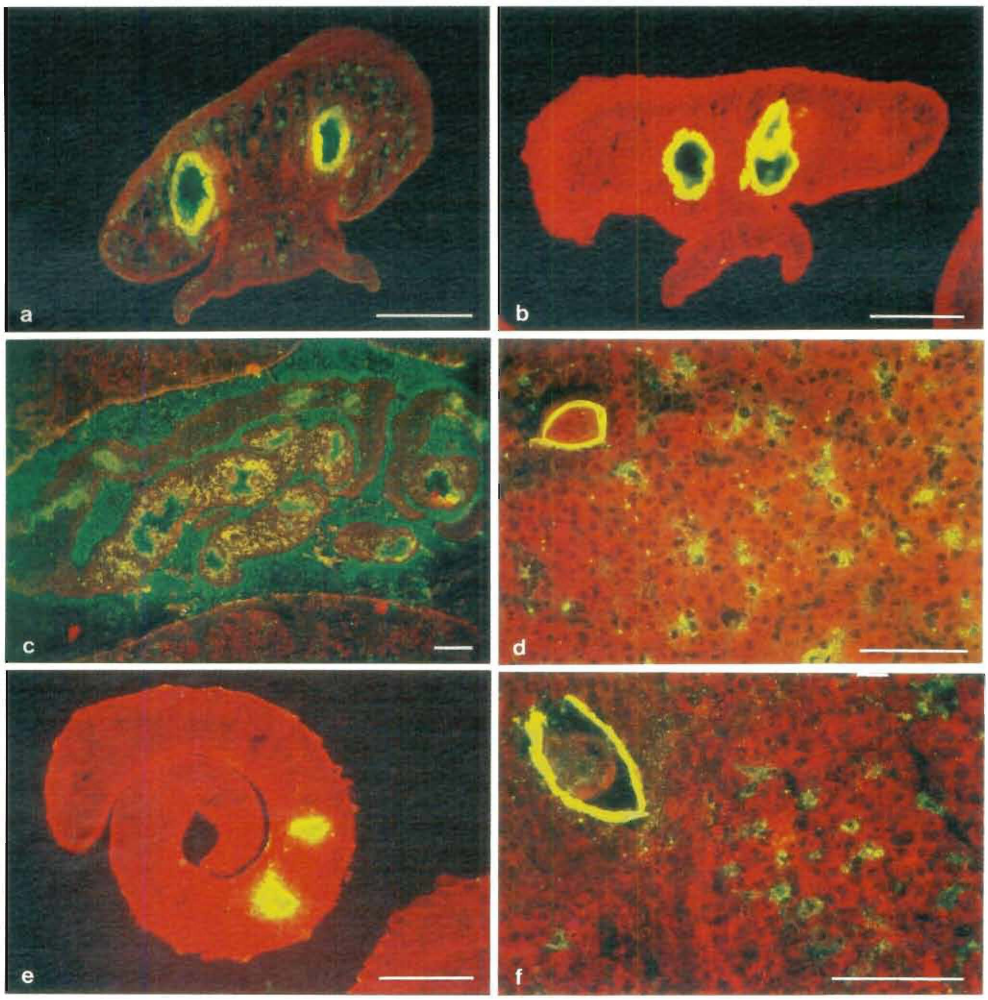


Figure 1. Immunofluorescence patterns of anti-CAA and anti-CCA McAbs on sections of adult *S. mansoni* worms fixed in Rossman's fixative (a,b,e) and on frozen sections of livers of *S. mansoni* infected hamsters (c,d,f). Sections were incubated with anti-CAA McAbs 25-9B10-A (a), 120-1B10-A (b), 54-5G10-A (c), 120-1B10-A (d), and with anti-CCA McAbs 54-4C2-A (e), 22-1B3-A (f). Bar = 100 μm.

Table 2. Monoclonal antibodies recognizing CAA.

McAb		IE				DIBA						IFA–F.S.							IFA–R.F.					
	Isotype	AWA	AWA–TCA	SEA	SEA–TCA	AWA	AWA–TCA	vomitus	SEA	SEA–TCA	Hi–TCA	HiUr–TCA	gut	parenchyma	tegument	free antigen	miracidium	egg shell	kupffer cells	gut	parenchyma	miracidium	egg shell	kupffer cells	liver nuclei
5–25–B	M	+	+	–	–	+	+	±	±	–	+	–	+	–	–	–	+	–	+	+	–	–	–	+	–
25–1E8–A	M	+	+	–	–	+	+	+	+	±	+	–	+	–	–	–	+	–	+	+	+	–	–	+	+
25–2B6–A	M	+	+	–	–	+	+	+	+	±	+	–	±	–	–	–	+	–	+	+	+	–	–	+	+
25–3D10–A	M	+	+	–	–	+	+	+	+	±	+	–	+	–	–	–	+	–	+	+	+	–	–	+	+
25–7C11–A	M	+	+	–	–	+	+	+	+	±	+	–	+	–	–	–	+	–	+	+	+	–	–	+	+
25–9B10–A	M	+	+	–	–	+	+	+	+	±	+	–	±	–	–	–	+	–	+	+	+	–	–	+	+
27–2E2–A	M	+	+	–	–	+	+	±	+	±	+	–	+	–	–	–	+	–	+	+	+	–	–	+	+
51–4B3–D	G3	+	+	–	–	+	+	±	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
51–4G5–A	G3	+	+	–	–	+	+	±	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
54–5C5–B	G3	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
54–5G10–A	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
114–4E10–A	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
120–1B10–A	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
120–1C2–C	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	–	–	–	+	+	–	–	–	+	–
120–1C11–C	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	-
141–2A9–A	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	–	–	–	+	+	–	–	–	+	–
145–2G1	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
147–1B1–A	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
147–1B4–A	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
147–1C7–A	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
147–1D9–C	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
147–1F7–A	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
147–3C9–C	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
147–3G4–A	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–
147–4A4–A	G1	+	+	–	–	+	+	+	–	–	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–

Table 3A. Monoclonal antibodies recognizing CCA.

McAb			IE				DIBA							IFA–F.S.							IFA–R.F.					
	number in pool	Isotype	AWA	AWA–TCA	SEA	SEA–TCA	AWA	AWA–TCA	vomitus	SEA	SEA–TCA	Hi–TCA	HiUr–TCA	gut	parenchyma	tegument	free antigen	miracidium	egg shell	kupffer cells	gut	parenchyma	miracidium	egg shell	kupffer cells	liver nuclei
22–1B3–A		M	+	+	+	+	+	+	±	+	+	–	–	+	–	±	–	+	+	+	+	–	+	+	–	–
22–2C4–A	2	M	+	+	+	+	+	+	+	+	+	±	–	+	–	–	–	–	+	+	+	–	+	+	+	–
22–2F10–A	2	M	+	+	+	+	+	+	+	+	+	±	–	+	–	+	–	–	+	+	+	–	+	+	–	–
22–5G11–E		M	+	+	+	+	+	+	+	+	+	±	–	+	–	±	–	–	+	+	+	–	–	+	–	–
24–1B2–A		M	+	+	+	+	+	+	+	+	+	±	–	+	–	±	–	+	+	+	+	–	+	+	–	–
24–2E5–A		M	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	+	+	+	+	–	+	+	–	–
24–4D7–A	2	M	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	+	+	+	–	+	+	–	–
51–4B9–A		G3	+	+	–	+	+	+	±	–	–	–	–	+	–	–	–	–	–	+	+	–	–	–	±	–
54–1F6–A		M	+	+	–	+	+	+	+	–	–	±	–	+	–	–	–	+	–	+	+	–	–	–	–	–
54–4C2–A	3	G1	+	+	–	–	+	+	+	–	–	±	–	+	–	–	–	–	–	+	+	–	–	–	±	–
54–5C10–A		G3	+	+	–	+	+	+	±	–	–	–	–	+	–	–	–	–	–	+	+	–	–	–	±	–
54–6E12–A		G1	+	+	–	–	+	+	±	–	–	–	–	+	–	–	–	–	–	+	+	–	–	–	±	–
114–1D11–B		M	+	+	–	–	+	+	+	+	+	±	–	+	–	–	–	+	–	+	+	–	–	+	–	–
114–1H12–A		G1	+	+	–	–	+	+	±	±	±	±	–	+	–	–	–	–	–	+	+	–	–	–	–	–
114–2B4–C		M	+	+	–	–	+	+	±	–	–	–	–	+	–	–	–	+	–	+	+	–	–	±	–	–
114–2G6–A		M	+	+	–	–	+	+	±	±	±	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–
114–2H9–B		M	+	+	–	–	+	+	±	–	–	–	–	+	–	–	–	+	–	+	+	–	–	–	+	–
114–3A7–A		M	+	+	–	–	+	+	±	±	±	–	–	+	–	–	–	+	–	+	+	–	–	±	–	–
114–3A12–A	2	M	+	+	–	–	+	+	±	–	–	±	–	+	–	–	–	+	–	+	+	–	–	–	–	–
114–3C5–B		M	+	+	–	–	+	+	±	±	–	±	–	+	–	–	–	+	–	+	+	–	–	–	–	–
114–4A1–A	2	M	+	+	+	+	+	+	+	+	+	±	–	+	–	–	–	+	+	+	+	–	–	+	–	–
114–5A10–A		G1	+	+	+	+	+	+	+	+	+	±	–	+	–	–	–	±	±	+	+	–	+	+	–	–
114–5F12–A	7	M	+	+	–	–	+	+	±	–	–	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–
128–1G3–B		M	+	+	–	–	+	+	±	±	–	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–
179–4B1–A		G3	+	+	+	+	+	+	±	+	+	–	–	+	–	–	–	–	–	+	+	±	–	–	–	–
180–1A10–A		G1	+	+	+	+	+	+	±	±	±	–	–	+	–	–	–	–	–	+	+	+	+	–	–	–
180–1D9–A	2	M	+	+	+	+	+	+	±	+	+	±	–	+	–	–	–	+	+	+	+	–	–	+	+	–
180–1D11–B		G1	+	+	–	–	+	+	±	±	±	±	–	+	–	–	–	–	–	+	+	–	–	–	±	–
204–3D8–A		M	+	+	–	–	+	+	±	–	–	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–
218–2F10–A	3	M	+	+	–	–	+	+	±	–	–	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–
257–2C1–A	2	M	+	+	–	+	+	+	±	–	–	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–
257–3D5–A	2	M	+	+	–	–	+	+	±	±	±	–	–	+	–	–	–	+	–	+	+	+	+	–	–	–
257–4A8–A		M	+	+	–	–	+	+	±	±	±	±	–	+	–	–	–	+	–	+	+	–	–	–	–	–
259–4E10–A		M	+	+	–	–	+	+	±	–	–	–	–	+	–	–	–	+	–	+	+	+	+	–	–	–
273–2C7–A		M	+	+	+	+	+	+	±	+	+	–	–	+	–	–	–	+	+	+	+	+	+	–	–	–
273–2D11–A		M	+	+	+	+	+	+	±	+	+	–	–	+	–	–	–	+	+	+	+	–	+	–	–	–
274–1B8–A		M	+	+	–	–	+	+	±	–	–	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–
8–3C10		M	+	+	–	–	+	+	±	–	–	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–

Table 3B. McAbs with reactivity patterns identical to that of the representative McAbs in Table 3A.

representative McAb	identical McAbs	representative McAb	identical McAbs	representative McAb	identical McAbs
22-2C4-A	22-2C9-A	114-4A1-A	114-2B12-A	180-1D9-A	180-1E6-A
22-2F10-A	22-3A1-A	114-5F12-A	114-2D8-B	218-2F10-A	218-2G11-A
			114-3G5-A		218-5C9-A
24-4D7-A	24-5G1-A		114-4D9-A		
			114-4B3-A	257-2C1-A	257-5B5-A
54-4C2-A	54-3H5-A		115-5B5-A		
	54-6G1-B		114-5G3-C	257-3D5-A	257-4E12-A
114-3A12-A	114-3F6-A				

The results of an IFA on different life cycle stages of the parasite, as determined with two representative anti-CAA and two anti-CCA McAbs, is given in Table 4. Incubation of whole cercariae, schistosomula or 5-days old lung stage worms resulted in staining of the tegument, both with anti-CAA and with anti-CCA antibodies. On frozen sections of these stages, gut fluorescence was generally detectable both with anti-CAA and anti-CCA antibodies.

Table 4. Immunofluorescence reaction of anti-CAA and anti-CCA McAbs on different life cycle stages of *Schistosoma mansoni*. Parts of organism or section that are not found positive with either McAb are not mentioned in the table.

McAb	Life cycle stage																
	cercaria				schistosomulum					lung worm				adult worm			egg	
	O[a]	T	G	F	O	T	W	G	P	O	T	W	G	T	G	P	L	S
anti-CAA																		
25-9B10-A	+[b]	-	+	-	+	-	+	+	-	+	+	-	+	-	+	+	+	-
120-1B10-A	+	-	+	-	+	-	-	+	-	+	-	+	+	-	+	-	-	-
anti-CCA																		
22-2F10-A	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+
54-5C10-A	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+	-	-	-

[a] Explanation of abbreviations: O = whole organism, fluorescence at tegument; other abbreviations concern sections of the different parasite life cycle stages: C = flame cell-like structures; G = gut; L = gland-like structure; P = parenchyma; T = tegument; S = (egg)shell; W = whole body

[b] symbols represent: - = no fluorescence; +, +, + = average fluorescence intensity 1,2,3

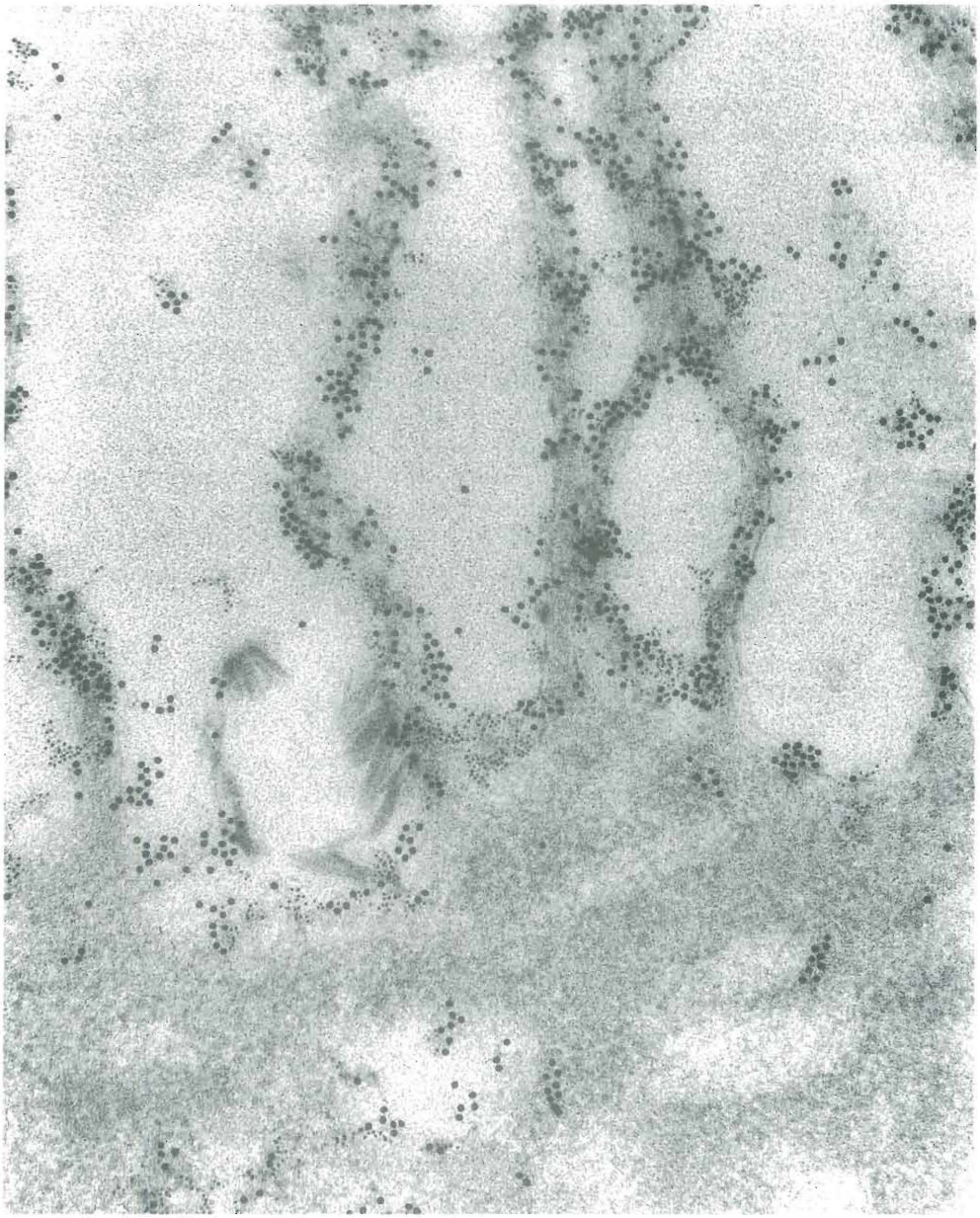


Figure 2. Ultrastructural localization of CAA and CCA on a section of syncytium of gut of *S. mansoni*, using an immunogold procedure with anti-CCA McAb (54-4C2-A) labeled with 15 nm colloidal gold and with anti-CAA McAb (54-5G10-A) labeled with 7 nm colloidal gold. Final magnification: 67 000 ×.

Ultrastructural localization on Lowicryl-embedded *S. mansoni* worms, using a double staining with anti-CAA and anti-CCA antibody each labeled with colloidal gold, clearly showed a strong and same recognition of antigen at the level of the syncytium. Both antigens were present in vesicles in the syncytium and, more strongly, at the level of the microvilli protruding into the lumen of the gut (Fig. 2).

Immunoprecipitation reactions

In immunoelectrophoresis (IE), all anti-CAA McAbs strongly reacted with AWA and with AWA-TCA, showing the characteristic anodic precipitate. With AWA-TCA the precipitate was slightly more anodic than with AWA (Fig. 3). In addition, with AWA the precipitate often showed an extension of the arc in a half-circle around the well, which was not the case with AWA-TCA. Anti-CAA McAbs never showed any reactivity with either SEA or SEA-TCA in IE. Anti-CCA McAbs all reacted with AWA and AWA-TCA showing a slightly cathodic precipitate, which was often a bit fuzzy (Fig. 3). Twenty-four out of the 55 anti-CCA McAbs also reacted with SEA, or in particular with SEA-TCA. The anti-SEA-TCA precipitate had a position which was slightly more anodic than the precipitate with AWA-TCA.

In immunodiffusion (ID) against AWA-TCA, but also against Hi-TCA and HiUr-TCA, anti-CAA and anti-CCA McAbs showed a complete lack of recognition of the same repeating epitopes (Fig. 4). Furthermore, ID clearly demonstrated that both CAA and CCA possess several repeating epitopes, as visualized by lack of identity in immunoprecipitation lines of *e.g.* different anti-CCA antibodies.

Dot immunobinding assay (DIBA)

While immunofluorescence and immunoprecipitation studies already clearly indicated that CAA and CCA each presented a number of repeating epitopes recognized by different groups of McAbs, additional characterization in a DIBA against a panel of antigens was carried out. These included adult worm antigen and soluble egg antigen, vomitus of adult worms, and the TCA-soluble fractions of serum and urine of infected hamsters (all antigens or infections: *S. mansoni*). The observed reactivity patterns again showed that on both antigens McAbs recognized a number of different epitopes (Tables 2, 3, and Fig. 5).

All anti-CAA McAbs were reactive with AWA, AWA-TCA, worm vomitus and the TCA-soluble fraction of serum of infected hamsters. No reaction was found with urine of infected hamsters. IgM McAbs of fusions 25 and 27, generated against *S. japonicum* antigens, also reacted with SEA and to a lesser degree with SEA-TCA.

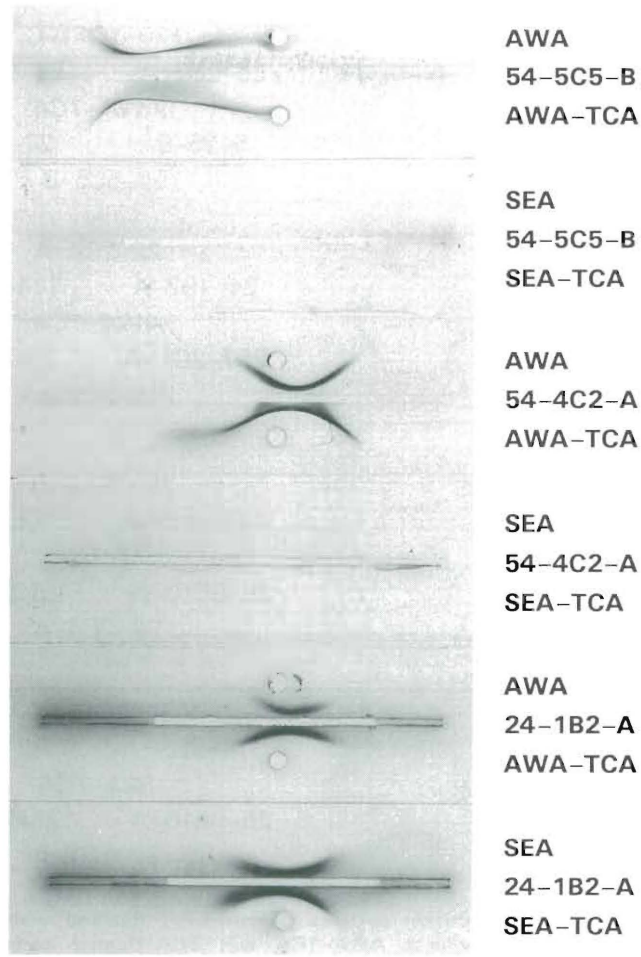


Figure 3. Immunoelectropherograms of anti-CAA McAb (54-5C5-B) and anti-CCA McAbs (54-4C2-A, 24-1B2-A) against AWA, AWA-TCA, SEA and SEA-TCA.

The reactivity pattern of anti-CCA McAbs in the DIBA was more diverse than that of anti-CAA McAbs. All anti-CCA McAbs reacted with AWA, AWA-TCA and with worm vomitus. In general, the reaction with worm vomitus was lower than that of anti-CAA antibodies. No reactivity was observed with urine of infected hamsters, while – in contrast to anti-CAA antibodies – anti-CCA antibodies gave no, or only a weak reaction with the TCA-soluble fraction of serum of infected hamsters. Many of the anti-CCA McAbs recognized SEA and SEA-TCA, or in five cases SEA-TCA only. Although the reactivity with egg antigens was particularly strong and uniform for all IgM monoclonals of fusions 22, 24 and 273, some monoclonals of IgG1 and IgG3 isotype also showed anti-egg antigen reactivity.

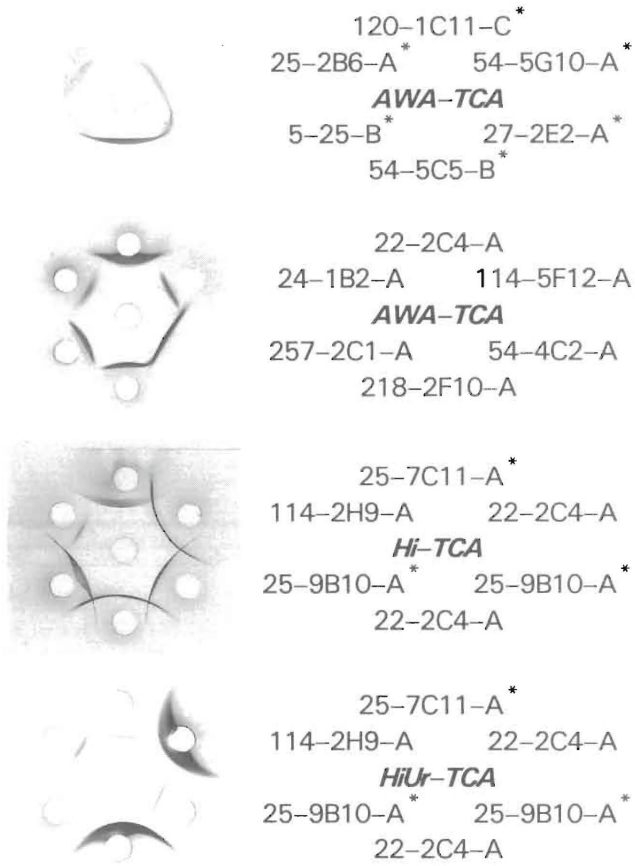


Figure 4. Immunodiffusion patterns of anti–CAA (marked with an asterix) and anti–CCA McAbs against AWA–TCA and TCA–treated serum and urine of *S. mansoni*–infected hamsters.

SDS–PAGE and Western blotting

After SDS–PAGE and Western blotting of AWA, anti–CAA McAbs showed only a weak smear ranging from 150 to 200 kDa, while anti–CCA McAbs showed a strong smear ranging from 70 to 200 kDa. When AWA–TCA instead of AWA was electrophoresed in a concentration that with regard to CAA and CCA was about one hundred–fold higher, anti–CAA McAbs now showed a stronger staining, again with a smear ranging from 150 to 200 kDa (Fig. 6). As within each of the two groups different McAbs showed identical patterns, not all monoclonals were tested.

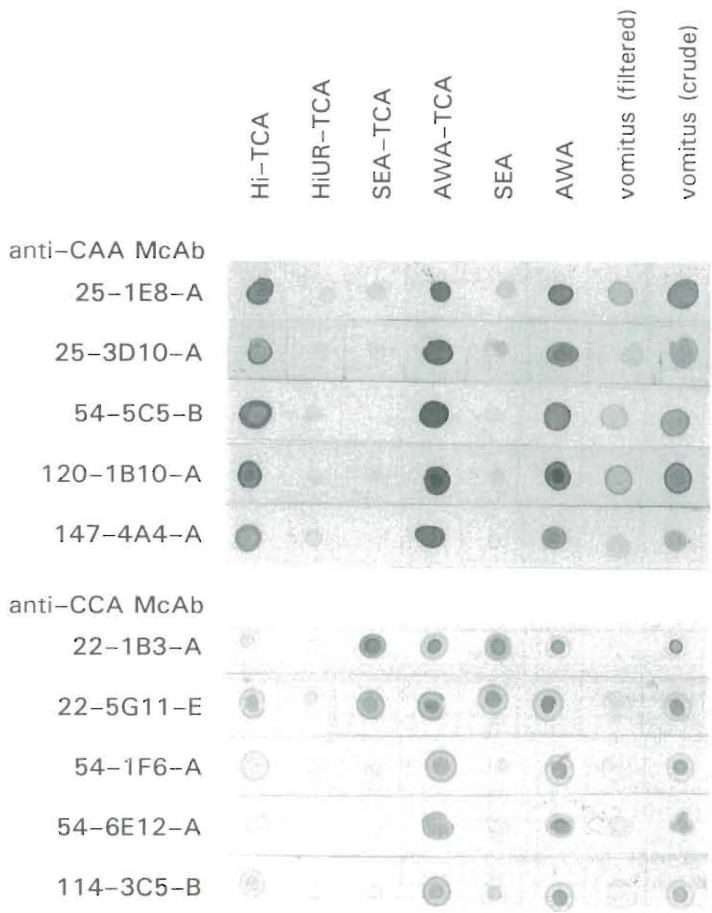
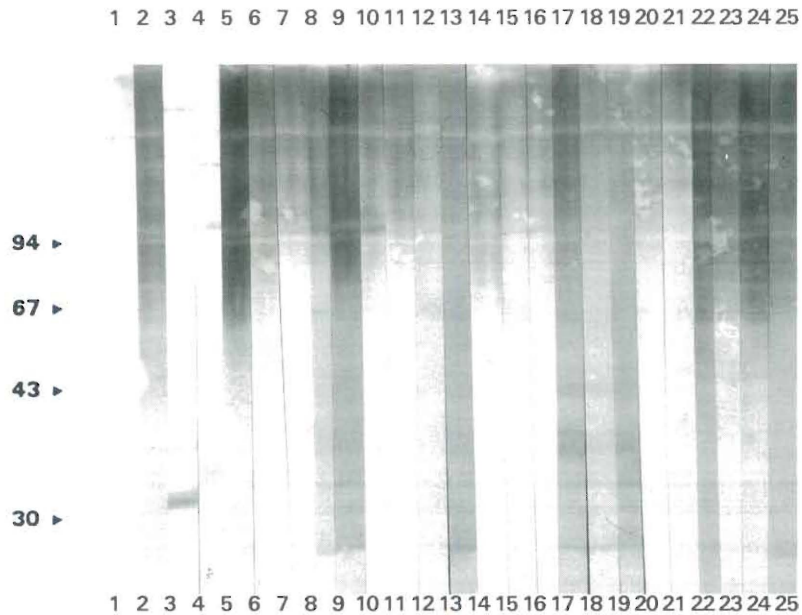


Figure 5. Dot immunobinding assay patterns of anti-CAA McAbs and anti-CCA McAbs against various antigen preparations.

Enzyme-linked immunosorbent assays (ELISA)

Although results with immunoprecipitation techniques, as shown above, clearly indicated that anti-CAA and anti-CCA McAbs do not recognize common repeating epitopes, experiments including combinations of an anti-CAA and an anti-CCA McAb in a sandwich ELISA resulted in a positive signal. To further elucidate this phenomenon, ELISA experiments were carried out using McAbs reactive with either antigen as capture and as detecting antibody, and as antigens AWA-TCA and purified CAA and CCA preparation (Fig. 7). In homologous assays, the related antigen was detected to a similar degree as AWA-TCA, while the unrelated antigen only gave a low reactivity. *i.e.*, an anti-CAA assay with anti-CAA capture and detection antibody sensitively detected AWA-TCA and



strip nr.	McAb	strip nr.	McAb	strip nr.	McAb
1	negative ascites	8	24-2E5-A	17	114-4A1-A
2	24-1B2-A	9	24-4D7-A	18	114-4B3-A
3	154-3G8-A (anti-32 kDa)	10	24-5G1-A	19	114-4D9-B
		11	114-2B12-A	20	114-5F12-A
4	154-3G8-A (anti-36 kDa)	12	114-2D8-B	21	128-1G3-B
		13	114-2H9-A	22	180-1D9-C
5	22-2C4-A	14	114-3A7-A	23	180-1E6-A
6	22-2F10-A	15	114-3F6-A	24	54-4C2-A
7	22-3A1-A	16	114-3G5-A	25	180-1D11-B

Figure 6. Immunoblot-patterns of a number of anti-CCA McAbs and controls after electrophoretic separation of AWA on an 8% polyacrylamide gel.

CAA, but not CCA, and vice versa for an anti-CCA assay.

In an ELISA with anti-CAA capture antibody and anti-CCA detection antibody, overall reactivity with all antigen preparations was reduced, with the highest reactivity with AWA-TCA and slightly lower reactivity for CAA, and again a lower but still clearly measurable reactivity with CCA. When anti-CCA antibody was used as capture antibody and anti-CAA antibody as detection antibody, a relatively strong reaction was seen with AWA-TCA and with CAA, but no reactivity with CCA. These data strongly suggest that on CCA no or only few "CAA-epitopes" are present, but that "CCA-epitopes" are present on CAA.

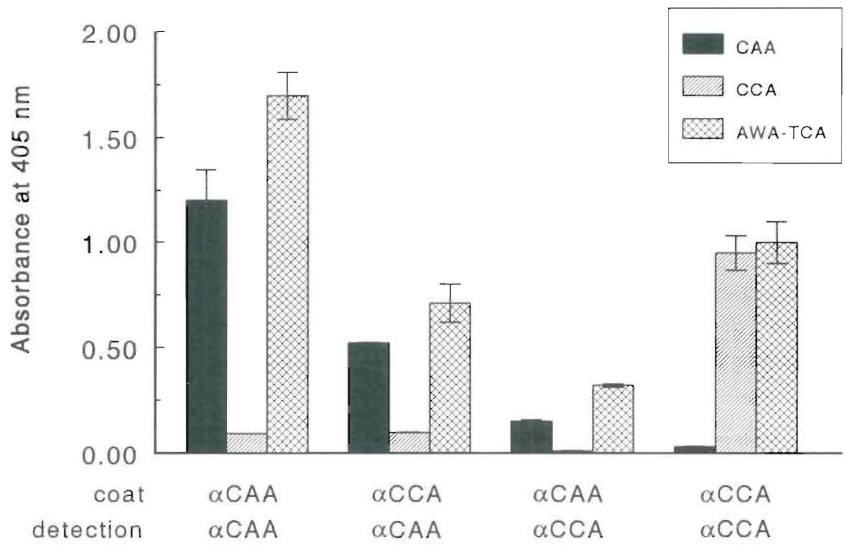


Figure 7. Reactivity of AWA-TCA, immunopurified CAA and CCA preparations in 4 different antigen-capture ELISA's. The absorbances at 405 nm of duplicate wells containing 100 ng/ml AWA-TCA or an equivalent amount of CAA or CCA, as measured in the standard CAA-capture or the CCA-capture ELISA.

Discussion

Using mouse hybridoma technology, a large number of fusions were carried out to generate panels of McAbs against two major diagnostic antigens for schistosomiasis, CAA and CCA. The resultant large and unique panels of monoclonals reactive with various epitopes allow us to draw a number of general conclusions.

Firstly, relevant hybridoma's could be isolated from mice which had been infected with *S. mansoni*, *S. haematobium* or *S. japonicum*, and from mice which had been immunized with circulating antigen-enriched antigen preparations or with crude adult worm antigen. This shows that independently from "natural" or artificial antigen presentation, both antigens are immunogenic in the mouse,and provides corroborating evidence for the fact that both antigens are common to the genus *Schistosoma*. Immunization with CCA, *e.g.* in the rabbit, fails to produce satisfactory polyvalent antisera, one of the reasons why originally [17,18] hybridoma technology was applied to this area of research.

From a considerable number of fusions performed for anti-CAA and anti-CCA McAb production, nineteen were positive in generating one or more relevant

monoclonals. It is interesting to note (a) that, with a few exceptions, one and the same fusion yielded either anti-CAA or anti-CCA antibodies, (b) that anti-CCA McAbs were generated about twice as often as anti-CAA McAbs, and (c) that against both antigens a strong isotype restriction existed. While against CAA primarily IgG1 and to a lesser degree IgM and IgG3 antibodies were found, 80% of the antibodies against CCA were IgM, with only occasionally IgG1 and IgG3 antibodies. In line with these findings, other anti-CCA antibodies which have been described in literature are indeed of IgM isotype [2,34,39].

Both circulating antigens were originally described, and named after their behaviour in immunoelectrophoresis, based on the reaction of a specific antibody with repeating epitopes on the antigens. In view of the fact that other characterization techniques that are generally applied for proteins, like Western blotting, gave unsatisfactory results, immunoelectrophoresis remained a crucial step in defining anti-CAA and anti-CCA McAbs. Only those antibodies reactive with repeating (carbohydrate) epitopes were selected for the present panels, although in our collection also a number of McAbs are available which react with AWA-TCA in a sandwich-ELISA using anti-CCA McAb as capture antibody. Such antibodies are possibly directed against a non-repeating epitope, *e.g.* on the protein backbone of CCA, or may alternatively be directed against other antigens but recognize an epitope that is common to CCA.

The combination of different immunological techniques employed in this study clearly demonstrates that both CAA and CCA possess several repeating epitopes which are immunogenic. For CAA, we recently described that the major carbohydrate chains have a novel polysaccharide structure, consisting of a branched disaccharide repeating unit, containing 2-acetamido-2-deoxy-β-D-galactopyranose and β-D-glucopyranuronic acid [5]. The present analysis shows that CAA contains at least five different repeating epitopes, although the reactivity pattern of the majority of the anti-CAA McAbs is strikingly similar, suggesting one major immunogenic repeating epitope. None of the epitopes possibly occurs as a repeating epitope on egg antigens, as shown by the lack of reactivity of anti-CAA monoclonals with SEA in immunoelectrophoresis. A number of IgM anti-CAA monoclonals reacted with SEA preparations in the DIBA and with miracidia in the IFA, however, which might indicate that the epitopes on egg antigen(s) are non-repeating or that they are repeating but only present in small numbers, not detectable by the relatively insensitive immunoprecipitation techniques IE and ID.

The glycoconjugate CCA appears to have a more diverse immunogenic structure. Our recent carbohydrate analysis showed that the major carbohydrate fraction of this antigen comprises a population of polysaccharides, containing Lewis x repeating units (→3)Galβ(1→4)[Fucα(1→3)]GlcNacβ(1→) [48]. In addition, a minor carbohydrate fraction was found, consisting of disaccharides to hexasaccharides

having a Galβ(1→3)GalNac-OL core in common. It is difficult to define exactly how many different epitopes are present on CCA, as additional immunological tests might divide groups of monoclonals now apparently reacting with the same epitope. About ten different repeating epitopes appear to be present, however. More often than with CAA, such epitopes appear to be present also on egg antigen(s), as shown by DIBA and IFA. In contrast to CAA, the marked reactivity of anti-CCA McAbs against SEA in IE, shows that such epitopes are also repeating.

This reactivity of many IgM anti-CCA monoclonals with carbohydrate egg antigens may be biologically relevant. We have shown before, that already early in human schistosome infections high levels of IgM antibodies against CCA are demonstrable [22]. As several publications [23,28] have reported that IgM antibodies reactive with carbohydrate egg antigens may block binding of protective IgG reactive with schistosomulum surface antigens, the formation of anti-CCA IgM antibodies might play a role in the parasite's evasion of immune mechanisms of the host. A second important role might be due to the fact that anti-CCA antibodies recognize repeating Lewis x units as found on *e.g.* circulating neutrophils [46]. The formation of these anti-schistosome antibodies might thus lead to the induction of "auto-immunity" against granulocytes.

The presence of different repeating epitopes on both antigens has implications for immunodiagnosis. Firstly, our studies on CAA [12,16] and those of us [13] and others [3,24] on CCA, have shown that one monoclonal can successfully be used both as capture and as detection antibody in sandwich immunoassays. Alternatively, a combination of two monoclonals, as described by us for an anti-CCA ELISA may result in enhanced sensitivity. Secondly, the fact that CCA presents a poly-Lewis x structure may result in false-positive results in uninfected individuals, particularly when urine samples of individuals with urinary tract infection, associated with leucocyturia, are tested (unpublished observations). This finding might explain the relatively high cut-off value which has to be used in the CCA-ELISA [32]. The application of anti-CCA McAbs reactive with other epitopes, which might overcome this problem, is now under study. Thirdly, an interesting and important finding is the fact that anti-CCA monoclonals also react with CAA, as shown in this study by ELISA-methodology and independently by structural analysis [5]. Carbohydrate analysis has shown that the CAA protein backbone carries at least one CCA-chain. The fact that, as shown by the complete absence of a precipitate in immunoelectrophoresis, anti-CCA monoclonals are not able to precipitate CAA, *i.e.* are not reactive with a repeating epitope, may be due to the complete lack of repetition of the epitope or due to steric hindrance. For immunodiagnosis using sandwich immunoassays this finding, as corroborated by the ELISA-data of the present study, would suggest a sufficient "mutual exclusion" of CAA-

and CCA-assays. Particularly the anti-CAA assay, using a McAb that recognizes a unique carbohydrate structure [5], shows a very high specificity.

For the characterization of the produced monoclonals a set of different techniques were used. Crucial information for initial selection was obtained by immunofluorescence and subsequent IE. The immunoblot gave little information due to the smeared electrophoretic pattern of the primarily carbohydrate antigens. The DIBA allowed rapid classification of McAb activity, but an absence of reactivity against some preparations in this assay should be interpreted with care. For example, no reactivity at all was observed with anti-CCA monoclonals against urine of infected hamsters, while a strong reactivity with the same monoclonals against the same antigen preparation was evident in immunodiffusion. The lack of reactivity in the DIBA must therefore be explained by non-binding of the antigen (in urine possibly present purely as carbohydrate chain or as antigen fragment) to the nitrocellulose. The fact that serum of infected hamsters reacts well in DIBA with anti-CCA antibodies, may be explained by the apparent presence of the antigen in serum in the form of immune complexes, as shown by Qian *et al.* for sera from *S. japonicum* infected individuals [43,44].

An interesting observation was that McAbs against both CAA and CCA which in the adult worm showed primarily gut fluorescence, were reactive with CAA- and CCA-epitopes which were developmentally expressed on a.o. the surface of developing schistosomes. This finding is of interest in view of the recent study of Köster and Strand [29] on two different fuscose-containing carbohydrate epitopes which showed a different and developmentally regulated expression. One of their McAbs, 128C3/3, showed a binding to the excretory systems of adult worms strikingly similar to that described by us for a McAb reactive with a circulating egg antigen [6,40,41], which also recognized epitopes present on the surface of cercariae. The second McAb studied by Köster and Strand, McAb 504B1, bound to the Le^x epitope [29] and thus might be similar to our anti-CCA McAbs. Both McAb 540B1 and our anti-CCA McAbs were strongly reactive with antigens in the gut, although a binding like that of 504B1 to the surface of the adult schistosome was only observed for a restricted number of the anti-CCA McAbs.

In conclusion, it can be stated that the production of panels of monoclonal antibodies against CAA and CCA, which was originally undertaken to select McAbs optimally performing in diagnostic immunoassays, has allowed us to study the epitope repertoire of these two antigens. It was conclusively shown that both antigens, with as major immunodominant structures polysaccharides containing repeating disaccharides (CAA) or trisaccharides (CCA) [5,48], also possess a number of different multiple repeating epitopes. Both antigens are continuously secreted in relatively large quantities from the gut of the adult

worms into the circulation of the host and are strongly immunogenic. As such, the developmentally regulated expression of both CAA and CCA and the fact that several epitopes are common to CCA and the egg would imply a complicated interaction of these two antigens with the immune defense mechanism of the host.

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