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

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Chapter 4

Schistosoma mansoni: analysis of monoclonal antibodies reactive with gut-associated antigens

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Chapter 4

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Abstract

The analysis of a series of monoclonal antibodies (McAbs) developed in our laboratory against gut-associated antigens of *Schistosoma mansoni* is described. It was found that McAbs that recognized epitopes of antigens in the gut and on the eggshell were mainly of the IgM isotype; these epitopes are likely to be carbohydrate in composition. Of a number of McAbs that were reactive with antigens important to the human humoral immune response 75% appeared to be reactive with the circulating cathodic antigen.

Introduction

Antigen analysis has played an essential role in elucidating the immunological interaction between *S. mansoni* and its host. Most of the research on antigens of the (developing) worm has been focused on tegument antigens, as these antigens appear to be primary targets for immune attack and are thus potential candidates for vaccines [26,28,33,37,39]. In the humoral immune response of the host, an early and strong reactivity is also observed against a number of gut-associated antigens [11,18,31,36]. Such antigens are regularly released by the schistosome into the circulation of the host when the parasite regurgitates the undigested contents of the gut.

Some gut-associated antigens have been well characterized, and they basically belong to two main groups: (1) parasitic proteolytic enzymes, e.g. cathepsin B; and (2) the highly glycosylated circulating anodic and cathodic antigen (CAA and CCA; [16,18,32]). The proteases were studied using immunoblotting and histochemical techniques and their molecular weights ranged from 28 to 36 kDa [5,10-12,18,22,39]. CAA and CCA are named after their electrophoretic

mobility and are antigens with a high content of carbohydrates, remaining soluble and stable in 7.5% trichloroacetic acid (TCA). They also retain their immunoreactivity after treatment with Rossman's fixative [18,30,31], a saturated solution of picric acid in ethanol. This fixative denatures protein epitopes, whereas carbohydrate structures are conserved. The function of CAA and CCA is not known, but they are thought to play a role in the digestion of food [13] or to protect the intestinal cells against low pH, antibody [30], or proteolytic secretions [9]. Two additional gut-associated circulating antigens have been described: M antigen (by Carlier et al. (1980) [9]), which is supposed to be identical to CCA, and an antigen that is also excreted into urine (by Appriou et al. (1986) [4]).

Among a large panel of monoclonal antibodies (McAbs) showing gut-associated reactivity in the immunofluorescence assay (IFA) on adult schistosomes, a number of McAbs recognized antigens different from those described above. Many of these McAbs recognized epitopes that persisted after treatment with Rossman's fixative and showed reactivity with egg antigens. Using these McAbs in the present study, we attempted to define gut-associated antigens additional to the two groups described above.

A total of 71 McAbs was selected that recognized gut-associated antigens as shown by the IFA on cryostat sections of livers from hamsters infected with *S. mansoni*. McAbs recognizing schistosome gut proteases, CAA, or CCA were excluded. The 71 McAbs were studied using the following techniques: IFA on paraffin sections of adult male worms fixed in Rossman's fixative, dot immunobinding assay (DIBA), inhibition DIBA in which binding of McAbs was inhibited by a pool of sera from *S. mansoni*-infected patients, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of homogenized adult worm antigen (AWA) followed by immunoblotting. A narrower selection was made using two criteria: strong gut-associated reactivity in the IFA and a positive reaction in the inhibition DIBA. Half of this group was characterized additionally using immunoelectrophoresis (IE) and enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Parasites and antigens

Schistosoma mansoni (Puerto Rico strain) adult worms were collected by perfusion of golden hamsters at 7 weeks after infection with 1 500 cercariae. Antigen preparations AWA, AWA-TCA, SEA, and SEA-TCA were prepared as previously described [18]. A

preparation of excretion and secretion antigens (ESA) was made as described by Rotmans *et al.* (1981) [34]. In short, worms were cultured in H-199 medium and antigens were isolated from the media. Concentration of the antigens and removal of small molecules (mol. wt., < 10 kDa) was done by ultrafiltration in an Amicon ultrafiltration cell (Amicon Corporation, Danvers, Ireland). After lyophilization, the ESA preparation was stored at −20°C. 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.

Monoclonal antibodies

Cell lines were prepared from different fusions. In general, mice were infected with 100–180 cercariae and spleen cells were fused with SP2/0 myeloma cells after 8–15 weeks. 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 2 weeks, tested in the IFA for reactivity, and stored at −20°C until its use. For those hybridomas of which no ascitic fluid was made, the supernatant of the cell culture or the ammonium sulfate precipitate thereof was used in the assays. Isotypes of the McAbs were generally determined in IFA or DIBA using anti-mouse isotype-specific (fluorescein isothiocyanate (FITC) or peroxidase (PO) conjugates.

Immunofluorescence assay

The IFA was carried out on either sections of frozen livers from infected hamsters, sections of adult male worms fixed with Rossman's fixative, or both [30,31]. Slides were incubated with diluted samples of the McAb solutions in phosphate-buffered saline (PBS; 0.035 M phosphate, 0.15 M NaCl, pH 7.8), washed, and incubated with a FITC conjugate of rabbit-anti-mouse immunoglobulin antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) diluted 1/50 in PBS containing 0.1 mg Evan's Blue/ml. The slides were observed with a Leitz Dialux 20EB fluorescence microscope equipped with the appropriate filter combination for FITC fluorescence. Negative controls consisted of fresh culture medium or ascitic fluid from mice injected with the SP2/0 myeloma line only. Fluorescence was interpreted visually on an ordinal scale ranging from 0 to 3 and was expressed as the average intensity of at least four observations carried out at different positions in the sections.

DIBA and inhibition-DIBA

In the DIBA, solutions of AWA, AWA-TCA, SEA, SEA-TCA, ESA and WV in PBS (1 mg/ml) were used as antigens. In short, 1 μl antigen solution was spotted on strips of nitrocellulose paper (0.45 μm, Schleicher und Schuell, Dassel, FRG), heated to 80°C for 1 h, blocked with bovine serum albumin (BSA), and successively incubated with McAb solutions, with a PO conjugate of rabbit-anti-mouse immunoglobulin antibodies

(Dakopatts, Glostrup, Denmark) and with a substrate solution (1.4 mM diaminobenzidine, 1.4 mM 4-chloro-1-naphthol, and 4 mM H_2O_2). The intensities of the dots were interpreted visually on a scale of 0 to 3.

In the inhibition-DIBA [2] the strips were incubated with a 1/30 dilution (in PBS) of a pool of human sera with a high antibody titer against *S. mansoni* (average titer, >1024 as measured in the routinely used IFA on male worms fixed in Rossman's fixative [17]), prior to the McAb incubation. As a negative control, a pool of negative control sera (titer, <8) was used. For purposes of comparison, McAbs that were known to recognize CAA, CCA or gut proteases were tested in the inhibition DIBA.

SDS-PAGE and Western-blotting

AWA was electrophoretically separated in the Laemmli system [25]; 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 [41] at 4°C on a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, Mass., 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 that were then incubated with the McAb solutions diluted in 5% dried skimmed milk in PBS, washed, incubated with the PO conjugate of rabbit-anti-mouse immunoglobulin in 5% dried milk in PBS, washed again, and finally incubated with the substrate solution as described above.

Immunoelectrophoresis

IE was carried out as described by Capron *et al.* (1965) on microscope-slides covered with an agarose gel (1% SeaKem Agarose; FMC Corporation, Rockland, Mass., U.S.A.) in 0.08 M Veronalbuffer (pH 8.2) [7]. AWA (0.8 mg) or AWA-TCA (0.4 mg) was used as the antigen, and undiluted ascitic fluid or purified antibody from culture supernatant served as the antibody. Electrophoresis was performed for 3 h at 50 V and 2.5 mA per slide. After diffusion, washing and drying, the slides were stained with Amido-Black (Merck, Darmstadt, FRG).

Two-site ELISA

The reactivity of McAbs with CCA was determined in a two-site ELISA. In short, polyvinylchloride (PVC) microtitration plates (Flow Laboratories, Irvine, UK) were coated with an anti-CCA McAb (IgG3 isotype), blocked with BSA, and incubated first with a concentration series of AWA-TCA and next with the McAb to be tested, which was then finally detected with a PO conjugate specific for the isotype of the McAb (which was either IgM or IgG1). A 5-aminosalicylic acid solution containing H_2O_2 was used as

the substrate. Between incubation steps plates were thoroughly washed with a 20-fold diluted PBS solution.

Data analysis

To test whether the isotype distribution patterns of McAbs recognizing certain target epitopes would differ significantly from the pattern expected for the whole panel, a χ^2-test (with Yate's correction for continuity) was applied. The tests were run on an IBM/XT compatible computer using the SPSS/PC+ software (SPSS, Chicago, Ill., USA).

Results

McAbs reactive with gut-associated antigens were screened with an IFA assay on frozen sections of adult worms. A total of 71 McAbs showing an average intensity value of >0.5 were considered as positive and were selected for further studies. In all, 54 McAbs also showed gut-fluorescence on sections of worms fixed in Rossman's fixative. Fluorescence was not restricted to the gut only; depending on the McAb many other tissue components gave a positive reaction. Typical examples are given in Figs. 1–3, showing the reactivities of several McAbs on sections of frozen livers from *Schistosoma mansoni*-infected hamsters, containing both adult worms and eggs (Figs. 1, 3) and on sections of adult male worms fixed in Rossman's fixative (Fig. 2).

Table 1. IFA: numbers of McAbs[a] recognizing common epitopes present on various tissues of cryostat sections of livers of *Schistosoma mansoni*-infected hamsters.

	McAb reactive with						
and	P[b] IgM–IgG	M IgM–IgG	T IgM–IgG	ES IgM–IgG	Mir IgM–IgG	EBL IgM–IgG	K IgM–IgG
P	16 – 6						
M	5 – 3	5 – 4					
T	13 – 3	5 – 3	15 – 11				
ES	15 – 0	4 – 0	13 – 0	20 – 5			
Mir	10 – 6	4 – 4	9 – 9	11 – 5	16 – 30		
EBL	8 – 0	3 – 0	6 – 1	12 – 1	6 – 2	12 – 2	
K	13 – 3	4 – 1	12 – 9	18 – 2	11 – 17	11 – 1	21 – 28

[a] whole panel consisted of 71 McAbs (26 IgM and 45 IgG) showing gut-associated fluorescence on cryostat sections.

[b] *P*, parenchyma; *M*, muscles; *T*, tegument; *ES*, eggshell; *Mir*, miracidium; *EBL*, eggblebs; *K*, Kupffer cells

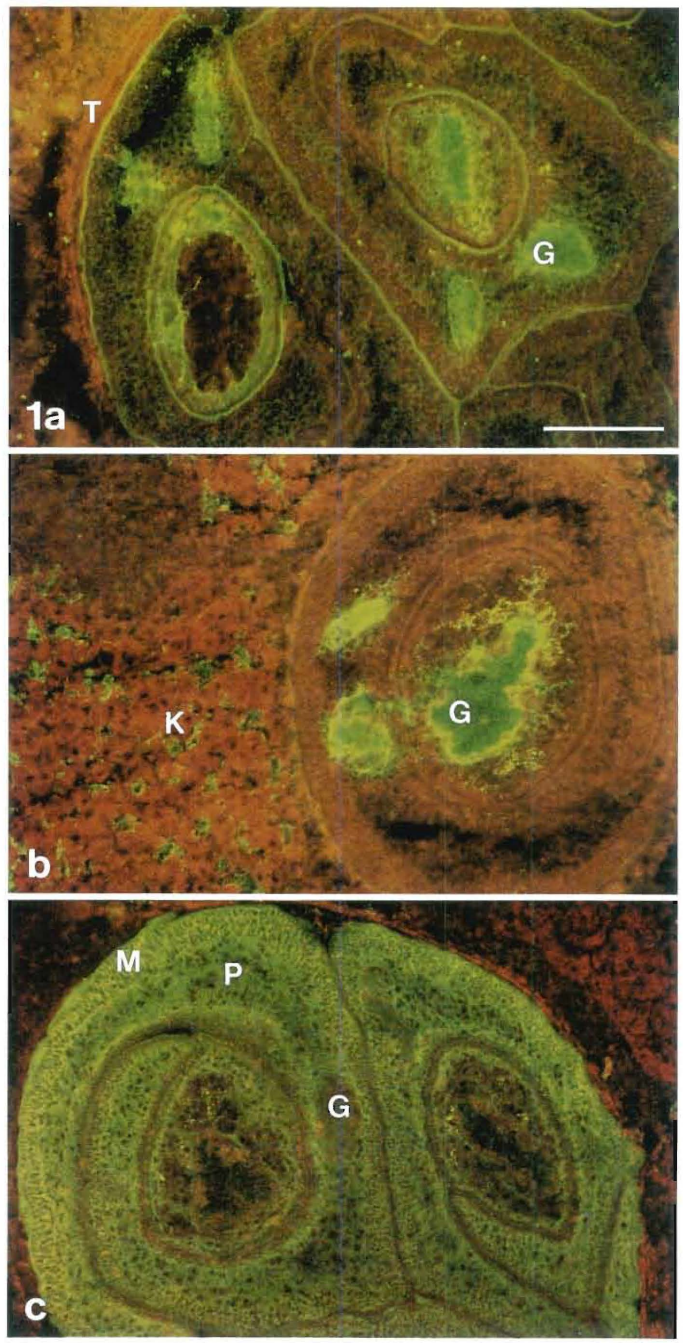

Figure 1a–c. IFA: recognition of common epitopes on frozen sections of ***Schistosoma mansoni*** adult worms in infected hamster liver. Sections were incubated with McAbs a 100-3C6-A, **b** 114-5G3-C, and **c** 128-2G1, followed by a FITC conjugate of rabbit-anti-mouse Ig antibodies. Fluorescence patterns were observed a in the gut (*G*) and on the tegument (*T*); b in the gut (*G*) and in Kupffer cells (*K*); and c in the muscles (*M*) and in the parenchyma (*P*; note the weak fluorescence in the gut). Bar = 100 μm

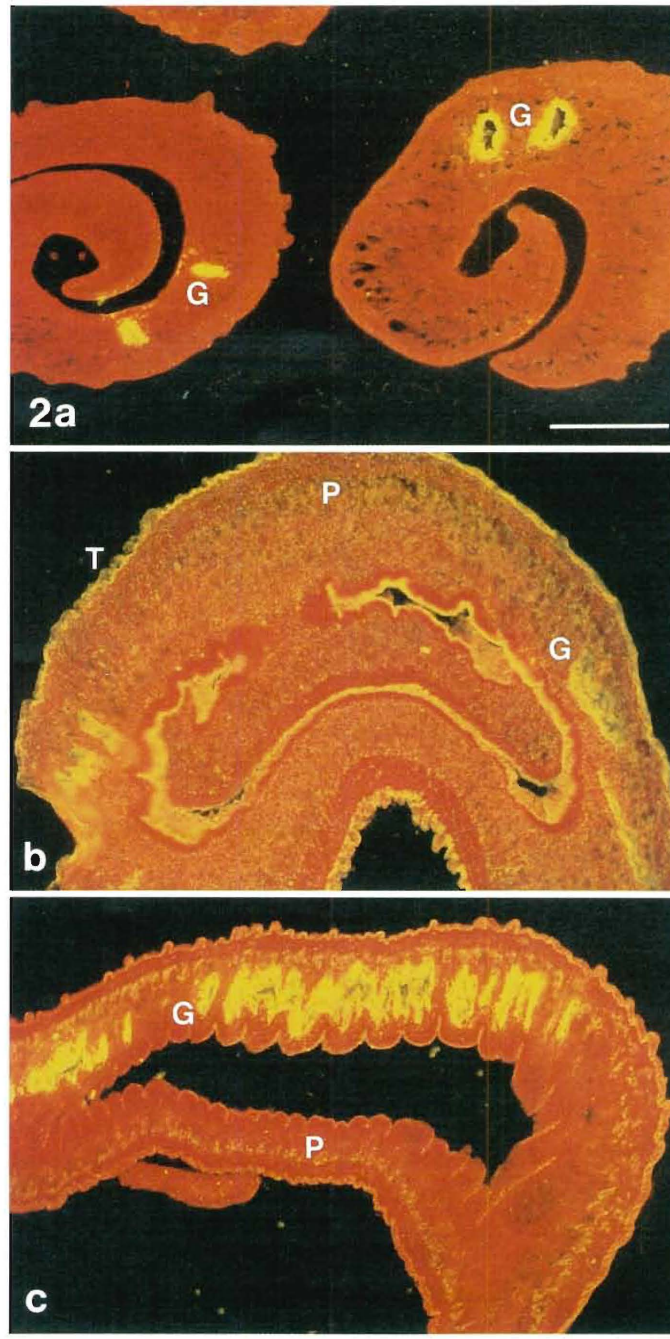

Figure 2a–c. IFA: recognition of common epitopes on sections of *Schistosoma mansoni* adult male worms fixed in Rossman's fixative. Sections were incubated with McAbs **a** 114-5G3-A, **b** 99-2B9-A, and **c** 99-1G3-A followed by a FITC conjugate of rabbit-anti-mouse Ig antibodies. Fluorescence patterns were observed **a** in the gut (*G*) as well as **b**, **c** in the gut (*G*), in the parenchyma (*P*, weak), and on the tegument (*T*). Bar = 100 μm

Although gut fluorescence was prominent (Figs. 1, 2), a positive reaction was also observed on the tegument (Figs. 1a, 2b), and in the worm parenchyma and muscles (Figs. 1c, 2b, c). Some McAbs showed reactivity with Kupffer cells (Fig. 1b). In Fig. 3 fluorescence can be seen in different components of the schistosome egg, including the eggshell (Fig. 3a, clearly discernable from autofluorescence), dispersed antigen associated with the eggshell (Fig. 3a), antigen released from the eggs in the form of blebs (Fig. 3b), and in the miracidium inside the egg (Fig. 3c).

Table 2. IFA: numbers of McAbs[a] recognizing common epitopes present on various tissues of paraffin sections of *Schistosoma mansoni* male worms fixed with Rossman's fixative.

	McAb reactive with		
and	G[b] IgM–IgG	P IgM–IgG	T IgM–IgG
G	23 – 31		
P	12 – 2	14 – 4	
T	10 – 2	9 – 2	10 – 4

[a] as described in table 1.
[b] *G*, gut; *P*, parenchyma; *T*, tegument

Table 3. DIBA: numbers of McAbs[a] recognizing various *Schistosoma mansoni* antigen preparations.

	McAb reactive with					
and	A[b] IgM–IgG	AT IgM–IgG	W IgM–IgG	ST IgM–IgG	E IgM–IgG	WV IgM–IgG
A	10 – 17					
AT	7 – 15	7 – 16				
S	5 – 1	2 – 0	20 – 8			
ST	6 – 2	3 – 1	19 – 4	20 – 6		
E	10 – 16	7 – 15	14 – 4	14 – 5	19 – 24	
WV	6 – 10	6 – 10	4 – 3	4 – 3	8 – 14	8 – 14

[a] as described in table 1.
[b] *A*, AWA; *AT*, AWA–TCA; *S*, SEA; *ST*, SEA–TCA; *E*, ESA; *WV*, worm vomitus

Most of the McAbs had the IgM (26 McAbs) or IgG1 isotype (37 McAbs). In addition, 7 IgG3 McAbs and 1 IgG2a McAb were found. The reactivity with epitopes common to gut-associated antigens and to other antigens was analyzed by cross-tabulation of IgG and IgM antibody reactivities in Tables 1

and 2. In Table 1, the numbers of McAbs that on cryostat sections of livers of *S. mansoni*-infected hamsters reacted with gut-associated antigens and, simultaneously, with epitopes in the parenchyma, tegument, eggshell, and other tissues are recorded. Likewise, in Table 2 the numbers of McAbs are given that showed simultaneous recognition of epitopes on antigens in the gut, parenchyma, muscles, and other tissues of worms that were fixed in Rossman's fixative. Epitopes on the eggshell were recognized mainly by antibodies of the IgM isotype (20 of 26 IgM vs only 5 of 45 IgG, $\chi^2 = 28$, $P < 0.00005$). Epitopes commonly present on gut and tegument antigens as well as on eggshell antigens appeared to be exclusively of the IgM isotype ($\chi^2 = 24$, $P < 0.00005$). Altogether, 46 McAbs recognized the miracidium, showing an isotype distribution similar to that of the whole panel.

The reactivity of the McAbs to six different antigen preparations (AWA, AWA-TCA, SEA, SEA-TCA, ESA, and WV) was tested in the DIBA. In Table 3 the cross-reactivity of the IgG and IgM McAbs with the various antigen preparations is recorded. Egg antigens were primarily recognized by IgM McAbs ($\chi^2 = 21$, $P < 0.00005$), whereas isotype-distribution patterns comparable with that of the whole panel were observed for reactivity toward whole-worm antigen preparations and ESA or WV. All McAbs that recognized WV, also recognized ESA.

On an immunoblot of adult worm antigen separated by SDS-PAGE on a 12% polyacrylamide gel, only three McAbs showed a distinct band, at respectively 28 (double band), ~28 kDa (a broad band), and 42 kDa, respectively. In all, 16 McAbs showed a dark smear at a high molecular weight of ca. 60-200 kDa), indicating reactivity with a polydisperse antigen (data not shown).

In the inhibition DIBA, a typical example of which is given in Fig. 4, the binding of 31 McAbs to AWA or AWA-TCA was strongly inhibited by prior incubation with a pool of sera from infected humans. Control experiments revealed that McAbs reactive with CAA or gut proteases, were not inhibited by incubation with the infected human serum pool, whereas McAbs reactive with CCA were strongly inhibited.

A total of 31 McAbs showed both strong inhibition in the inhibition DIBA and a strong gut fluorescence in the IFA and 16 of these were randomly selected for further study. If not present, ascitic fluid preparations were made, and these were tested in IE, on an immunoblot, and in a two-site ELISA to determine CCA-reactivity. In the IE, 2 McAbs clearly recognized CCA (one example shown in Fig. 5). In the CCA-ELISA, 2 McAbs were negative for CCA, 2 McAbs of the IgG3 isotype could not be tested, and 12 McAbs, including the 2 IE-positives, were reactive with CCA (detection limits ranged from 1 to 100 ng CCA/ml). On the AWA immunoblot of an 8% polyacrylamide gel 11 of these 12 McAbs

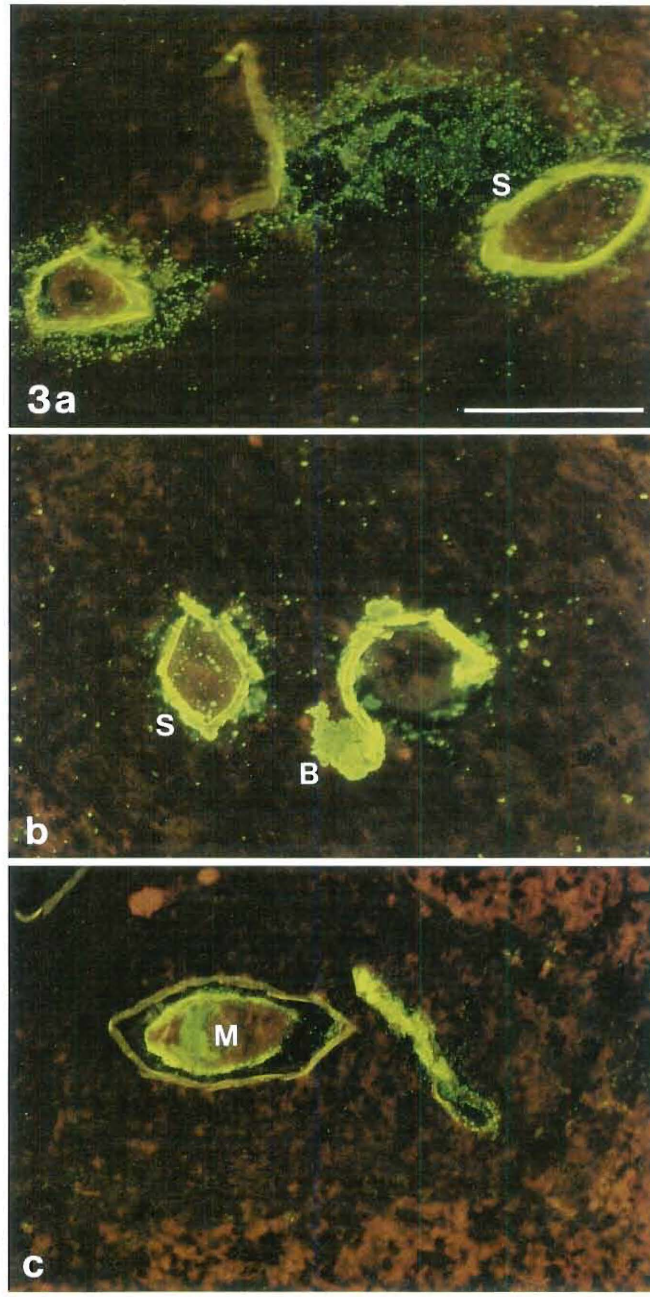

Figure 3a–c. IFA: recognition of common epitopes on eggs in frozen sections of *Schistosoma mansoni*-infected hamster liver. Sections were incubated with McAbs a 99-3E11-A, b 100-3C6-A, and c 54-5F5-A, followed by a FITC conjugate of rabbit-anti-mouse Ig antibodies. Fluorescence patterns were observed a on the eggshell (*S*), b in egg blebs (*B*) and on the eggshell (*S*), and c in the miracidium (*M*). Bar = 100 μm

ELISA-positive clearly and reproducibly identified a high molecular weight smear. This smear was identical to the one observed with approximately 95% of the previously characterized anti-CCA McAbs. The McAbs that tested negative in any of the three assays were also those that gave the relatively weakest inhibition in the inhibition DIBA.

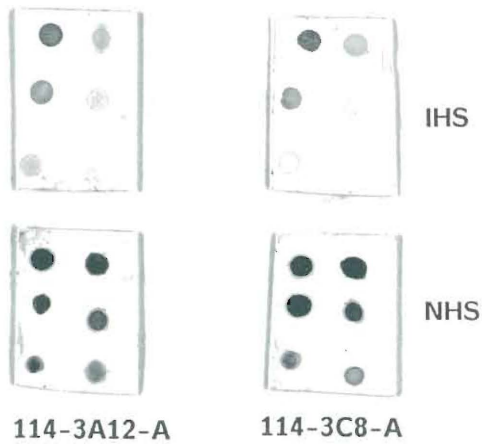

Figure 4. Inhibition DIBA of McAbs 114-3A12-A and 114-3C8-A with infected and normal human serum (*IHS, NHS*). Three quantities of AWA (*left*) and AWA-TCA (*right*) have been spotted (*from top to bottom*: 1, 0.25, and 0.06 μg), followed by consecutive incubations with human serum pools, with McAb solutions, and PO conjugate of rabbit-anti-mouse Ig antibodies, and with a substrate solution.

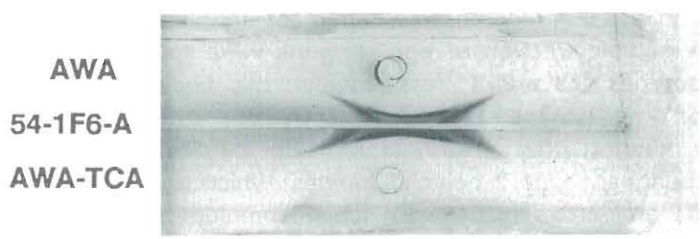

Figure 5. Immunoelectrophoresis of 0.8 mg AWA (*upper well*) and 0.4 mg AWA-TCA (*lower well*) against McAb 54-1F6-A (ascitic fluid). The anode is on the *left*.

Discussion

In our laboratory, most of the McAbs produced from *Schistosoma mansoni*–infected mice were of the IgM and IgG1 isotypes, which is in accordance with observations that in parasitic infections (e.g. *S. mansoni*), predominantly IgM, IgG1, and IgE levels are elevated [1,6,35,38]. IgE McAbs have never been found in our laboratory. It has also been reported that IgG1 is incremented in mice immunized with irradiated cercariae [20]. Although a large part of these antibodies might be nonspecific and are thought to be due to polyclonal B-cell activation [27,38], our results indicate that a majority of the *S. mansoni*–reactive McAbs that react with gut–associated antigens are of the IgM and IgG1 isotypes.

In the IFA carried out both on frozen sections of infected hamster livers and on paraffin sections of *S. mansoni* adult worms fixed in Rossman's fixative, many different recognition patterns were found, indicating that the McAbs recognized different epitopes. Common target epitopes were detected on antigens present in the gut of the worm and in the parenchyma or on the tegument. Gut–associated antigens recognized by 49 McAbs were also present in Kupffer cells, indicating that these antigens were probably released by the parasite into the host circulation [14].

From Tables 1 to 3 it is clear that significantly more IgM McAbs than IgG McAbs recognize epitopes of antigens in the gut and on the eggshell. In all, 18 of the 23 IgM McAbs that were reactive with gut–associated antigens on sections fixed in Rossman's fixative reacted with the eggshell on cryostat sections and with SEA or SEA–TCA in the DIBA as compared with only 7 of the 31 IgG McAbs ($\chi^2 = 14.3$, $P = 0.0002$). The observed resistance to the Rossman fixation procedure together with the TCA solubility strongly suggests that the common epitopes of the antigens in the gut and on the eggshell are carbohydrates.

McAbs that in the IFA recognized a tegument antigen and in the DIBA reacted with a TCA–soluble fraction of egg antigens were found only among the IgM and IgG3 isotypes (13 and 1, respectively); this finding is significantly higher than that for other isotypes ($\chi^2 = 17$, $P < 0.00005$). Although these numbers are small, they support the observations made by Mazza *et al.* (1990), that thymus–independent type 2 polysaccharides, which are particularly abundant in egg antigens and cross–react with schistosomulum surface antigens, exhibit antigen–directed isotype restriction in the form of IgM and IgG3 antibodies [29]. In our laboratory in general, significantly more IgM or IgG3 McAbs recognized egg antigens as compared with other isotypes ($n = 109$, $\chi^2 = 8.8$, $P = 0.003$, data not shown).

The inhibition DIBA was used to test whether the McAbs recognize antigens that are important to the human humoral immune response to the parasite. High titers of specific antibodies would result in a strong inhibition of the McAb binding to the antigens on the nitrocellulose strip. Using the criterion of a combination of positive gut fluorescence and inhibition by sera from infected humans, 31 McAbs were selected and, for practical reasons, half of them were additionally screened. In the further assays, ascitic fluid of those McAbs was used, as this normally contains higher concentrations of antibodies than culture supernatants. However, when ascitic fluid preparations were applied in the IE, two McAbs were found to react with CCA, an observation that was not previously made due to the low antibody titer of the preparations used in the IE.

Examination of the reactivity of anti-CCA McAbs on an AWA immunoblot of an 8% polyacrylamide gel showed that CCA was recognized as a high-molecular-weight (>60 kDa) smear of varying intensity. Carlier *et al.* (1980) found a similar pattern for their antigen M, which is supposed to be identical to CCA [9]. In all, 11 of the 16 selected McAbs showed this high-molecular-weight smear as well, suggesting that they recognized CCA. The reason why they tested negative in the IE against CCA may have been that a non-repetitive epitope was recognized or that the McAbs had a much lower affinity for CCA. A total of 12 McAbs (including the 2 showing a CCA-precipitating line in the IE) detected CCA in the CCA-ELISA, with lower detection levels ranging from 1 to 100 ng CCA/ml, indicating that the McAbs bound to CCA with different affinities or that the epitopes may have been masked by steric hindrance.

In conclusion, screening of the 71 selected McAbs did not result in the definition of important gut-associated antigens other than those that have previously been described. This indirectly adds to the importance of the above mentioned gut-associated antigens (CAA, CCA, and the gut proteolytic enzymes) to the humoral immune response. The observation in the inhibition DIBA that anti-CCA McAbs (but not anti-CAA or anti-gut proteases McAbs) were inhibited by sera from infected patients suggests that the humoral immune response to gut-associated antigens is predominantly against CCA. Deelder *et al.* (1989) found that in the IFA on adult worms fixed in Rossman's fixative, the positive reaction of human IgM antibodies was predominantly due to reactivity with CCA and not to reactivity with CAA or gut proteases [19]. Taken together, these findings are particularly interesting for several reasons: CCA, but also CAA, is involved in immunopathology as deposits of CCA-containing immune complexes are found in the kidney glomerulus of infected mice and humans [8,15,40]. Purified CCA, in contrast to CAA, is capable of activating complement in the absence of anti-schistosome antibodies, as are egg antigens [42]. This may imply a role for CCA in immune modulation, which has previously been suggested by Carlier *et al.* (1980) [9]. It has been reported that IgM antibodies reactive with

polysaccharide egg antigens may block a protective IgG response against schistosomulum surface antigens [3,21,23,24]. Whether this blocking activity can be attributed to the IgM McAbs recognizing epitopes on both highly glycosylated gut–associated antigens and egg antigens remains to be elucidated.

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