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Monoclonal antibodies reacting with *Schistosoma japonicum* eggs and their target epitopes

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Ten monoclonal antibodies (McAbs) raised to *Schistosoma japonicum* eggs could be assigned using several serological and immunochemical techniques to 3 groups. The McAbs, termed A, B and C-McAbs, apparently recognize carbohydrate epitopes that can be located on the same antigen molecule. The antibodies, generally of IgM isotype, are idiotypically related. They are distinct from another IgM McAb (Group D-McAb) the carbohydrate target epitope of which can also be associated with the epitopes of A, B and C-McAbs. The McAbs produce large vacuolated bleb reactions in the circumoval precipitin test (COPT) and target epitopes have different representations in various life cycle stages such as immature and mature eggs, male and female worms (including *S. mansoni*). Antigens affinity purified on columns containing A, B, C and D-McAbs stimulate proliferation of T cells from egg-sensitized mice and elicit DTH reactions in such mice. This raises the possibility that the target antigens of these carbohydrate-reactive monoclonal antibodies are immunopathologic and involved in egg-induced granuloma formation.

Key words: *Schistosoma japonicum*; Egg antigen; Carbohydrate epitope; Circumoval precipitin test; Immunoassay

Introduction

Studies on the egg antigens of *Schistosoma japonicum* parallel those in *S. mansoni*, and have been similarly directed towards the identification and characterization of egg antigens that are (a) immunopathologic (i.e. capable of sensitizing for and eliciting, granuloma formation) or (b) useful for immunodiagnosis. Methods employed in the studies of *S. japonicum* egg antigens include lectin and immunoaffinity chromatography (Carter and Colley, 1981; Long et al., 1981a,b; Tracy and Mahmoud, 1982; Norden and Strand, 1984; Mitchell et al., 1982; Sidner et al., 1987) and urea extraction (Tsang 1982). Monoclonal antibodies (McAbs) have been used (Cruise et al., 1981a,b; Mitchell et al., 1982; Miao et al., 1987). In this study, numerous McAbs were analyzed for their reactivity with *S. japonicum* eggs and twelve that gave circumoval precipitin reactions with eggs were selected for further characterization.

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Materials and Methods

Monoclonal antibodies

Details of the generation of the hybridomas using BALB/c mouse spleen cells and either P3–NS–1–Ag4–1 or Sp2/0–Ag–14 modified myeloma cells as well as selection assays, have been described (Cruise et al., 1981b; Mitchell et al., 1981a). The McAbs I.134–18–6, I.39–10–2, P.41–1–3, SEF.85–5–3, P.26–2–9 and TTO.45–8–8 have been characterized previously (Cruise et al., 1981a,b, 1983; Mitchell et al., 1981a, 1982, 1983a,b,c). The IM series of McAbs, not previously described, were prepared with spleen cells from BALB/c mice that received multiple injections of *S. japonicum* eggs in aqueous solution or with Freund's complete adjuvant. The S and J McAbs had been raised against lyophilized adult *S. japonicum* worms. As non-cross-reactive controls, McAbs Gib 13, which is directed against a filarial antigen (Forsyth et al., 1984), and WIC–108.3, which is directed against a glycolipid antigen of *Leishmania* (DeIbarra et al., 1982), were used and were gifts from Drs. K.P. Forsyth and E. Handman, respectively. Ammonium sulphate (50%) cuts of the ascites fluids from mice injected with the different hybridoma cells were used throughout this study. The presence of McAbs was detected by running small samples in agarose gels (Agarose Universal Electrophoresis Film, Corning, CA, U.S.A.) followed by staining with Coomassie blue and subsequent destaining. The relative mobility of the McAbs on agarose gels was noted. Four representative McAbs, viz. IM.31–6–6, IM.30–10–9, IM.12–6–1 and P.41–1–3, are referred to as A-, B-, C- and D-McAbs, respectively, and their epitopes as A, B, C, and D-epitopes.

Anti-idiotypic (Id) antibodies

The IM.12–6–1 McAb (C-McAb) (approximately 4 mg/ml) was coupled to an equal amount of keyhole limpet haemocyanin (KLH, Calbiochem, CA, U.S.A.) by adding 5 µl of 25% gluteraldehyde (electron microscopy grade, Taab Laboratories, Reading, U.K.) at three minute intervals with gentle stirring at room temperature (Seppala and Eichmann, 1979; Mitchell et al., 1982). The conjugates were dialyzed against mouse tonicity phosphate buffered saline (MTPBS, pH7.3, consisting of 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 0.15 M NaCl) and used to sensitize various inbred strains of mice first in Freund's complete adjuvant (FCA), followed by two aqueous boosts, both intraperitoneally (ip) and subcutaneously (sc) at a dose of 100 µg McAb. The mice were subsequently bled and the sera used in competitive radioimmunoassays.

Parasites and antigens

S. japonicum eggs were obtained from livers of rabbits (Smithers, 1960) that had been infected usually for 55 to 60 days, following exposure to cercariae obtained from crushed *Oncomelania hupensis quadrasi* snails collected from the field in Mindoro, Philippines or bred in the Manila laboratory. Adult worms were obtained by perfusion of the porto-mesenteric circulation (Mitchell et al., 1981b). This 'strain' of *S. japonicum* was used throughout this study. Egg antigens were prepared by mechanical homogenization or eggs (in MTPBS) in a glass tissue grinder with a

teflon-coated pestle, followed by sonication, centrifugation at 1 600, 15 600, 26 000 or 100 000 × *g*, and the supernatant stored at −70°C. The 100 000 × *g* supernatant is referred to as the soluble egg antigen (SEA) preparation. For Western blotting, the antigens were prepared in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62 mM Tris-HCl pH 6.8, 0.2% SDS, 50 mM dithiothreitol, 10% glycerol).

Circumoval precipitin test (COPT)

Lyophilized *S. japonicum* eggs were incubated with different dilutions of McAbs on a glass slide under a coverslip that was sealed with paraffin (Garcia et al., 1981). After incubation for 72 h at 37°C, COP reactivity was examined by light microscopy.

Radioimmunoassays (RIAs)

Wells of a polyvinylchloride plate (Dynatech, VA, U.S.A.) were coated with egg antigens for 3–4 h in a humidified box blocked with 0.5% bovine serum albumin in MTPBS (BSA/PBS) for 30 min and probed with McAb that had been radioiodinated by a modified chloramine T method (Greenwood et al., 1963) by J. Pye, D. Quilici or J. Patane of the Hall Institute to an activity of 370 kBq/μg (10 Ci/g). After incubation overnight, the washed wells of these direct binding RIA plates were counted in an autogamma counter.

Competitive radioimmunoassays (CRIAs) were as described earlier (Mitchell et al., 1981a). Wells of microtitre plates were coated with limiting dilution of egg antigens (as determined by previous direct-binding RIAs). After blocking and washing, unlabelled 'cold' McAbs were serially diluted in 25 μl of 0.5% BSA, 0.05% Tween 20 (Sigma, MO, U.S.A.) before 25 μl of radio-iodinated McAb (20 000–25 000 cpm) were added. The wells were counted 6–10 h after.

Two-site immunoradiometric assays (IRMAs) were performed in two ways. In the one-step IRMA, the wells of the first microtitre plate were coated with 50 μl of 10 μg/ml cold McAb in MTPBS, incubated in a humidified box for 3 h and afterwards blocked with 5% skim milk in MTPBS (BLOTTO, Johnson et al., 1984) for another hour. In parallel, wells of a second plate were coated with BLOTTO for 1 h, washed and SEA diluted five-fold serially in 25 μl of BLOTTO added followed by addition of 25 μl of radiolabelled McAb (approx. 40 000 cpm). After approximately 4 h, the first plate was washed and the contents of the second plate added to the first plate. The wells were counted after overnight incubation. The two-step IRMA is different from the first in that SEA was added into the wells coated with the cold McAb overnight, prior to washing, and adding radiolabelled McAbs. The sensitivities of these two variants of the same assay were compared (see Results section).

Western blotting

Egg (26 000 × *g* supernatant) and male worm antigens (26 000 × *g* supernatant) prepared in SDS-PAGE sample buffer were run over 13% gels and transferred to nitrocellulose filters according to the method of Burnette (1981).

After overnight transfer, the nitrocellulose filters were blocked with BLOTTO for 30–60 min, incubated with cold McAbs for 3–4 h and probed with affinity-purified

sheep anti-mouse immunoglobulin (Silenus, Victoria, Australia) that had been radioiodinated by the modified chloramine T method.

Determination of biochemical nature of target epitopes

Egg antigens (26 000 × *g* supernatant) were incubated in the dark with 20 mM sodium metaperiodate for 30 min at room temperature, in the presence of the protease inhibitor aprotinin (200 IU/ml, 'Trasylol' Bayer, F.R.G.). A control preparation was also incubated with the protease inhibitor. Both treated and control antigen preparations were dialyzed against MTPBS.

The same egg antigens (26 000 × *g* supernatant) were incubated with 3% trichloroacetic acid and 0.001% foetal calf serum at room temperature for 30 min in the presence of aprotinin. A control preparation similar to the above was set up. These were centrifuged for 15 min at 15 600 × *g* and the supernatant dialyzed against MTPBS.

Immunoaffinity purification of target antigens

McAbs (50% ammonium sulphate cuts of mouse ascites fluid) diluted in 0.5 M $NaHCO_3$ pH 8.0, 0.5 M NaCl (8 mg/ml) were coupled to CNBr-activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden) that had been swollen and washed according to the manufacturer's instructions. After excess McAbs were removed, the remaining active sites on the beads were blocked with 1 M glycine. The beads were incubated with four volumes of 0.25 M $NaHCO_3$ pH 8.0 for 1 h; afterwards the beads were sedimented and incubated in the same buffer with 0.06% gluteraldehyde added to reduce leakage of coupled proteins from the beads (Kowal and Parsons, 1980). These were then incubated with 1 M Tris-HCl pH 7.8 for more than 1 h to block the aldehyde groups before loading into columns ('Econocolumn', Biorad, CA, U.S.A.) followed by washing with alternating buffers of different pH, i.e. pH 4 and pH 8, before finally equilibrating with MTPBS. SEA was passed over two successive columns of Sepharose beads coupled with WIC–108.3 (WIC columns). The run through from the second column, termed SEA-RT, was then passed four times over a column of beads coupled with representative A, B, D and D-McAbs (ABCD column, see Results) at the proportion of 1:1:2:4 based on the relative abundance of the different epitopes as determined by IRMAs, or over another WIC column. The antigens were subsequently eluted with 0.1 M glycine-HCl, pH 2.6. The eluates, viz. ABCD eluate and WIC eluate, were concentrated by vacuum dialysis, and together with SEA and SEA-RT, used in two-step IRMAs with homologous cold and radiolabelled McAb combinations to determine the relative concentration of egg equivalents in the eluates.

T cell proliferation assay

T cells from C57BL/6 mice that had received two *S. japonicum* egg immunizations (the first in FCA) and FCA control mice were obtained by passing 1×10^8 spleen cells suspended in RPMI 1640 buffered with HEPES and supplemented with 5% FCS over a nylon wool column. After incubation for 45 min in a 37°C CO_2 incubation, the T cells were eluted from the column. Antigen-presenting cells were prepared by

subjecting spleen cells from naive mice to irradiation at 2500 rads. T cells (2×10^5) were incubated with antigen-presenting cells (5×10^5) and dilutions of SEA-RT and the immunoaffinity-purified antigens in the culture medium to which 20% CAS (supernatant from rat spleen cell cultures stimulated by concanavalin A, which serves as a source of lymphokines) was also added. Triplicate cultures of each antigen dilution were performed. The amount of WIC eluate used in each culture is equal to the volume of ABCD eluate. The antigens were sterilized by placing them near the ultraviolet light source in a laminar flow hood for 10 min. A ConA control was set up for each of the two sets of cultures, i.e. egg-sensitized and FCA control mouse T cells. The cultures were pulsed with 1 μCi [^{3}H]thymidine on day 3, and the cells harvested 16 h after. The radioactivity was counted in a liquid scintillation counter. Data were expressed as the mean (±SEM) of radioactivity counts minus the counts of cultures containing antigen-presenting cells and T cells, but no antigen.

Delayed type hypersensitivity (DTH) ear assay

C57BL/6 mice that had received two immunizations of *S. japonicum* eggs and FCA control mice were injected with the ABCD eluate (160 egg equivalents in 10 μl) intradermally in the left ear and an equal volume of MTPBS in the right ear. Ear swelling was measured with a microcaliper at 24 and 48 h. Data is presented as change in ear thickness (μm) which is the difference in the swelling between the left ear and the right ear. The same antigen preparation was injected into mice infected with *Leishmania major* in parallel with other positive control leishmania antigens.

Results

Classification of McAbs

Various of the 23 McAbs raised against *S. japonicum* and two other parasites (Table 1) were radioiodinated and were used first in direct binding RIAs to determine their reactivity with *S. japonicum* crude egg antigens (1 600 × *g* supernatant). These labelled McAbs were subsequently used in CRIAs with titrations of cold McAbs as inhibitors to determine relationships between individual McAbs in the battery. Data in Table 2 indicate that the IM series of anti-egg McAbs could be classified into three groups, designated A, B and C that were distinct from P.41–1–3 and SEF.85–5–3 (here designated group D) that were analyzed in detail previously (Cruise et al., 1981b; Mitchell et al., 1982; 1983a). Groups B and C McAbs are clearly related in that the binding of labelled B McAbs to crude egg antigens could be inhibited by group C McAbs although the reverse was not the case. The remaining McAbs in the battery that were negative or weakly positive in the circumoval precipitin test (COPT) (see Table 1) are quite distinct from groups A to D McAbs, according to results from CRIAs (data not shown).

In Table 1, a summary is provided of the COPT data, immunoglobulin isotype and agarose electrophoretic mobility of the McAbs in the battery. All group A McAbs showed a prozone effect at high concentrations in the COPT and had comparable electrophoretic mobility. It is likely that the IgM McAbs in this group are three isolates of the same antibody (see also Table 2). All 14 positive McAbs in the COPT

TABLE 1

Features of monoclonal antibodies (McAbs) reacting with *Schistosoma japonicum* eggs

McAb designation	Raised against	Group designation	Electrophoretic mobility	Isotype	72 h COPT result with dilutions[a]				Vacuolated precipitates in COPT
					Undiluted	1:10	1:100	1:1000	
IM.5–4–4	Egg	A	O/C	IgM	+	+ +	+	+	+
IM.30–6–6	Egg	A	O/C	IgM	+	+ +	+	0	±
IM.31–6–6	Egg	A	O/C	IgM	+	+ +	+	0	+
IM.5–7–7	Egg	B	C	IgM	+ +	+ +	+	0	±
IM.30–2–7	Egg	B	O/A	IgM	+ +	+ +	+	0	+
IM.30–10–9	Egg	B	O/A	IgM	+ +	+ +	+ +	+	+
IM.62–3–4	Egg	B	O	IgM	+ +	+ +	0	0	−
IM.12–3–2	Egg	C	O	IgM	+ +	+	0	0	±
IM.12–6–1	Egg	C	O	IgM	+ +	+ +	+ +	+	±
IM.117–6–5	Egg	C	O	IgM	+ +	+ +	+	0	±
P.41–1–3	P.w.	D	C	IgM	+ +	+ +	+	0	+
SEF.85–5–3	Egg	D	A	IgG_1	+ +	+ +	+	0	+
P.26–2–9	P.w.	–	O	IgM	0	+	+	0	−
S.137–3–24	AW	–	C	ND	+	+	0	0	−
IM.116–1–1	Egg	–	A/O	ND					
IM.116–2–5	Egg	–	A	ND					
I.134–18–6	AW	–	C	IgG_{2a}					
I.39–10–2	AW	–	O/C	IgM					
J.49–15–6	AW	–	C	ND		All negative			
J.49–13–2	AW	–	C	ND					
S.38–13–17	AW	–	O	ND					
S.38–10–1	AW	–	O	ND					
TTO.45–8–8	T.t.	–	ND	IgM					

Abbreviation key: For electrophoresis, O = remaining near origin; C = cathodal (slow) migration; A = anodal (faster) migration. For COPT, + + = numerous eggs positive; + = few eggs positive, and 0 = no COP reactions; vacuoles +, ± and 0 = most, few and no vacuolated precipitates, respectively. For antigens, AW = *S. japonicum* adult worm homogenate; Egg = *S. japonicum* eggs; P.w. = *Paragonimus westermani* adult worm antigen; T.t. = *Taenia taeniaeformis* oncosphere antigen; ND = not determined.

[a]50% ammonium sulphate cuts of ascites fluid at 5 mg/ml undiluted except for McAbs IM.5–7–7, IM.30–2–7, IM.12–3–2, S.137–3–24 and TTO.45–8–8 at 1 mg/ml.

produced bleb reactions rather than segmented precipitates (Cruise et al., 1981b; Hillyer and Pelley, 1980) and most of these blebs were vacuolated (Lewert et al., 1980) at high concentrations of McAb. Two McAbs, P.26–2–9 and S.137–3–24, did not cause vacuolated blebs and P.26–2–9 demonstrated a marked prozone effect in that no reaction was obtained at 5 mg/ml of McAb yet a clear positive reaction was noted at lower concentrations. These two McAbs are peculiar in that in CRIAs, *normal* mouse sera and control ascites fluids readily inhibit the binding of these to crude egg antigen. Thus it would seem that certain mouse immunoglobulins can cause non-specific bleb reactions of the small, non-vacuolated type in the COPT and that such a reaction need not necessarily represent a genuine antigen-antibody reaction.

TABLE 2

Classification of anti-egg McAbs into 4 groups on the basis of results of CRIAs using ^{125}I-labelled McAbs with egg antigens of *S. japonicum* on the plate

Cold inhibitor McAb	Percent inhibition of binding of labelled McAb						McAb group designation
	IM.5–4–4	IM.31–6–6	IM.5–7–7	IM.30–10–9	IM.12–6–1	P.41–1–3	
IM.5–4–4	>90–>90[a]	>90–>90	–	–	–	–	
IM.30–6–6	>90–>90	>90–>90	–	–	–	–	A
IM.31–6–6	>90–>90	>90–>90	–	–	–	–	
IM.5–7–7	–	–	>90–>90	50	–	–	
IM.30–2–7	–	–	>90–>90	>90–>90	–	–	B
IM.30–10–9	–	–	>90–>90	>90–>90	–	–	
IM.62–3–4	–	–	56–>90	33	–	–	
IM.12–3–2	–	–	36–44	46	70–>90	–	
IM.12–6–1	–	–	50–59	35–39	>90–>90	–	C
IM.117–6–5	–	–	53	30–41	69–>90	–	
P.41–1–3	–	–	–	–	–	88–>90	
SEF.85–5–3	–	–	–	–	–	73–>90	D

[a]Where a range of values is indicated, this denotes the percent inhibition of binding using cold inhibitor McAbs at 12.5 to 100 μg/ml. Single values represent inhibition of binding at 100 μg/ml cold McAb. Dashes denote background inhibition of binding at this highest concentration of cold McAb.

IM.31–6–6, IM.30–10–9, IM.12–6–1 and P.41–1–3, all IgMs and each capable of high inhibitory activity in CRIAs and therefore suggesting high binding avidity, were chosen as representatives for the A to D groups of McAbs, respectively. To facilitate presentation of data, these representative McAbs have been referred to as A-McAb, B-McAb, C-McAb and D-McAb, respectively, and the target determinants or epitopes to which they are directed as A, B, C and D-epitopes.

Idiotype (Id) relatedness

Relationships between the McAbs were also analyzed by anti-Id serology. Various strains of mice were immunized with C-McAb on KLH or KLH alone and sera used in CRIAs with labelled A, B, C and D-McAbs. Data in Table 3 indicate, rather surprisingly, that A, B and C-McAbs are idiotypically related as evidenced by inhibitory activity in sera from 4 of 8 mouse strains, some of which are the same Ig allotype as the BALB/c C-McAb. D-McAbs are apparently quite different from A, B or C-McAbs. We conclude that the combining sites of A, B, C and D-McAbs are different but that A, B and C-McAbs contain shared or related idiotope(s) that can be recognized by at least some mouse strains.

Combination of epitopes in SEA

The existence of the target epitope of A, B, C or D-McAb, in relation to homologous and heterologous epitopes on the same soluble egg molecule (or conglomerate of

TABLE 3

Relatedness of monoclonal antibodies assessed by anti-Id antibodies raised against C-McAb in CRIAs

Strain of mouse immunized	Immunogen	Reciprocal of nearest serum dilution resulting in 50% inhibition of binding of labelled McAb			
		A	B	C	D
CBA/H	C-KLH	>5120	>5120	>40960	<20
	KLH	<20	<20	<20	<20
C3H/He	C-KLH	2560	2560	40960	<20
	KLH	<20	<20	<20	<20
SJL/J	C-KLH	640	160	163840	<20
	KLH	<20	20	20	<20
C57BL/6	C-KLH	160	80	20480	<20
	KLH	<20	<20	<20	<20
WEHI 129/J	C-KLH	<20	80	81920	<20
	KLH	<20	160	40	<20
BALB/c.H-2^k	C-KLH	<20	40	40960	<20
	KLH	<20	<20	20	<20
BALB/c.H-2^b	C-KLH	<20	<20	20480	<20
	KLH	<20	<20	<20	<20
BALB/c	C-KLH	<20	<20	20480	<20
	KLH	<20	<20	<20	<20

molecules), and the relative frequencies of the epitopes in combinations, can be determined by two-site IRMAs. Two variants of IRMA were used in this study termed one-step and two-step IRMA. The IgM McAb, Gib 13, was used as the negative control because it has been successfully used in IRMAs to detect circulating antigens in sera of cattle infected with *Onchocerca gibsoni* (Forsyth et al., 1984) and in serum of humans suffering from Bancroftian filariasis (Forsyth et al., 1985). The results in Fig. 1, particularly using the two-step IRMA, suggest that A, B, C and D-epitopes can be found together but are not necessarily on the same molecule. A general estimation of the relative frequencies of the epitopes in combinations can be made by comparing the results from the two sets of IRMAs displayed in Fig 1. A-epitopes exist in homologous combinations in high proportion and in many fewer heterologous combinations with B and/or C and/or D-epitopes. In other words, the majority of B, C and D-epitopes are not connected with the A-epitope. Most of the

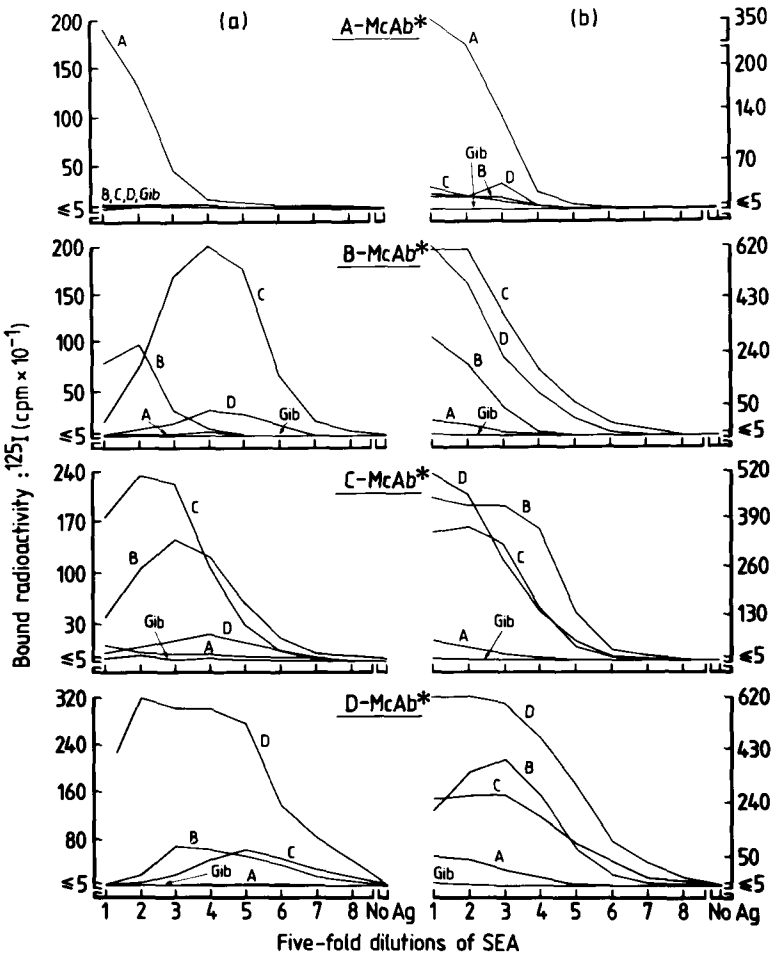

Fig. 1. One-step (a) and two-step (b) immunoradiometric assays (IRMAs) using ^{125}I-labelled A, B, C and D-McAbs, and corresponding cold monoclonals and an IgM McAb, Gib 13, coated to plates and reacted with five-fold dilutions of a soluble egg antigen preparation (SEA) commencing at 1:8. Diluent = BLOTTO.

D-epitopes are found in homologous combinations although an appreciable number of D-epitopes are in heterologous combinations with either or both B and C-epitopes, the two epitopes which have been suggested to be closely associated with each other on the basis of CRIA data (Table 2).

Stage specificity

Soluble immature egg antigens (100 000 × *g* supernatants) were prepared from eggs recovered from livers of rabbits harbouring infections not exceeding 30 days when miracidia would not yet have formed inside the eggs (Pesigan et al., 1958; Vogel, 1942). This was compared in CRIAs with an SEA preparation from 'mixed' eggs collected at 55 days of infection. The results indicate that the A-epitope is present only in mature eggs, that the D-epitope is present in minimal quantity in immature eggs but in greater abundance in mature eggs, whereas B and C-epitopes are present in both immature and mature eggs although their abundance in the former is much less than in the latter (data not shown).

To determine if the A, B, C and D-epitopes are present in the adult worm stages, with possible differences in combinations of epitopes, adult worm antigenic extracts (26 000 × *g* supernatants) were prepared from male adult *S. japonicum* worms (AWE-M) and used in two-step IRMAs. The results indicate that the A-epitope does not occur in AWE-M, either in homologous or heterologous combinations. B, C and D-epitopes are present in male worms, but the D-epitope seems not to be associated with B and C-epitopes in heterologous combinations in AWE-M, although B and C-epitopes are still consistently found to be associated with each other. The A-epitope is similarly not detectable in female worms (AWE-F) as might be expected for an antigen of mature eggs, whereas the other three epitopes are present (data not shown).

SEA and AWE-M were run on SDS-PAGE, blotted onto nitrocellulose, probed with the whole array of 12 individual McAbs belonging to the four groups (Table 2) and afterwards reacted with ^{125}I-labelled sheep anti-mouse immunoglobulin affinity-purified antibodies. In Fig. 2 the appearance of the immunoblots confirms the classification of McAbs according to CRIAs (Table 2) and IRMAs (Fig. 1). The A-epitope is not found in worm antigen extracts. The smears in the autoradiographs are characteristic of polysaccharides and glycolipids (Tsai and Frasch, 1982; Handman et al., 1984). A, B and C-epitope-containing bands of M_r > 94 000 and 59 000 can be seen in the A-McAb Westerns. The D-epitope-containing molecule(s) seem to be very large and were retained in the stacking gel. Differences between the Western blots of B-McAbs and C-McAbs are more apparent with the AWE-M than with SEA; nevertheless, a diffuse but distinct band of M_r > 94 000 is recognized by the McAbs of both groups.

Species specificity

SEA was prepared from *S. mansoni* eggs obtained from mice infected for 55 days and used in CRIAs. The results demonstrate that whereas B and C epitopes are both present in high amounts in *S. mansoni* eggs, A and D epitopes are both absent. The absence of the D-epitope is further confirmed by the inability of pooled sera from BALB/c mice infected for 11 weeks with *S. mansoni* to inhibit the binding of

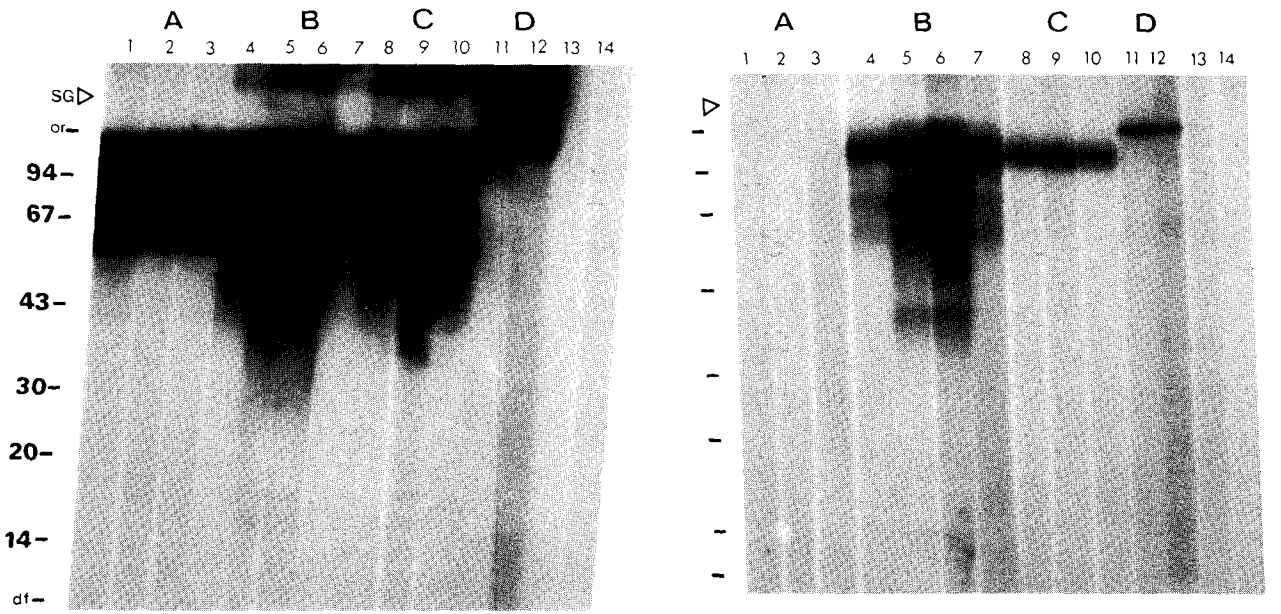

Fig. 2. Western blots of soluble egg antigens (SEA) (left panel) and male adult worm antigen extracts (AWE-M) probed with the groups of anti-egg McAbs and subsequently reacted with ^{125}I-labelled sheep anti-mouse immunoglobulin affinity-purified antibodies. Group A (1–3) = IM.5–4–4, IM.30–6–6, IM.31–6–6, respectively; Group B (4–7) = IM.5–7–7, IM.30–2–7, IM.30–10–9, IM.62–3–4, respectively; Group C (8–10) IM.12–3–2, IM.12–6–1, IM.117–6–5, respectively; and Group D (11–12) = P.41–1–3, SEF.85–5–3, respectively; 13 = IgM control McAb, Gib 13; 14 = negative background control.

radiolabelled D-McAb. Low levels of inhibition of radiolabelled A-McAb can however be detected in this serum suggesting that minimal amounts of A epitopes may be present in *S. mansoni* that can induce antibody responses (data not shown).

Biochemical nature of epitopes

SEA treated with sodium periodate when used in one-step CRIAs showed practically no inhibitory activity in the binding of labelled McAbs to their targets (Fig. 3). The supernatant taken from antigens after trichloroacetic acid (TCA) precipitation followed by centrifugation at $15\,600 \times g$ retained the inhibitory activity of the untreated samples. Furthermore, periodate-treated SEA, when used in two-step IRMAs with homologous or heterologous labelled and cold McAbs, only produced baseline radioactive counts (data not shown). These observations together with the appearance of the Western blots (Fig. 2) suggest that A, B and C-epitopes are carbohydrate in nature. The D-epitope has earlier been reported to be sensitive to periodate oxidation (Mitchell et al., 1983a). The reduction in the immunogenicity of the D-epitope after treatment with pronase, as determined by CRIAs with sera from C57BL/6 mice sensitized with the treated antigen, suggest that D-epitopes may also be linked to a protein moiety (data not shown).

T cell proliferation assay

Egg antigens containing any of the four A, B, C or D-epitopes were isolated by passing SEA through a Sepharose column, to which A, B, C and D-McAbs were coupled (i.e. ABCD column). To minimize non-specific binding of egg antigens to Sepharose and/or IgM antibody, SEA was passed first through two columns of Sepharose beads coupled with WIC-108.3. The runthrough (SEA-RT) was divided into two: one was passed through an ABCD column, and the other through another WIC-108.3 column and antigens eluted from these two columns (ABCD eluate and WIC eluate, respectively) were used in T cell proliferation assays.

Spleen cells were obtained from egg-sensitized and unsensitized C57BL/6 mice, and T cells enriched by passing the cell suspension through a nylon wool column. The cells were cultured in triplicate in the presence of irradiated antigen-presenting cells and dilutions of antigens. Fig. 4 presents the amount of radioactivity incorporated by T cells cultured in the presence of SEA-RT, ABCD eluate or WIC eluate and subsequently pulsed with [^{3}H]thymidine. The number of egg equivalents in the dilutions of SEA-RT and ABCD eluate were 1600, 320 and 64 as determined by two step IRMAs. The volume of WIC eluate used was equal to that of the ABCD eluate. Although no antigen titration pattern can be observed, the difference in the stimulatory effects of the three groups of antigens is obvious. SEA-RT has a higher stimulatory effect than the ABCD eluate suggesting that there are other T cell-stimulating egg antigens aside from the A, B, C or D-epitope-containing antigens. The stimulatory effect of the ABCD eluate cannot be ascribed readily to any other antigens that non-specifically adhere to IgM antibodies and/or Sepharose beads since the WIC eluate did not significantly stimulate T cells from egg-sensitized mice. A, B, C and D-epitopes can be detected in low amounts in the control (WIC) eluate but their concentration must be below the threshold for stimulation in the T cell assay.

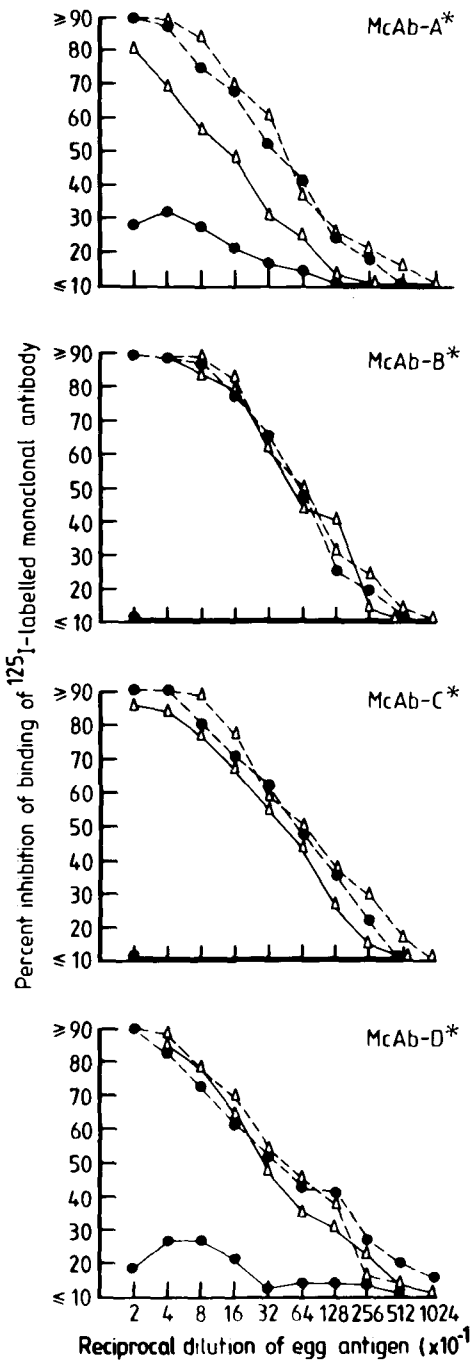

Fig. 3. Inhibition of binding of ^{125}I-labelled A, B, C and D-McAbs to solid-phase crude *S. japonicum* egg antigens (1600 × g) on the plate and using, as inhibitor, dilutions of egg antigens (26 000 × g) treated with sodium metaperiodate (●——●) or the non-precipitated material following trichloroacetic acid treatment (△——△). Respective treatment controls are indicated by broken lines.

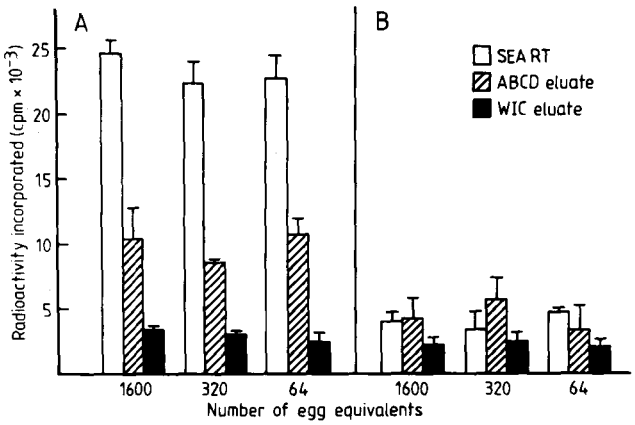

Fig. 4. Stimulation of T cell proliferative response, as determined by incorporation of [^{3}H]thymidine, with the SEA runthrough (SEA-RT) after passing through two WIC columns (open bars), the ABCD column eluate (hatched bars), or the WIC column eluate (solid bars). T cells were obtained from spleens of C57BL/6 mice that had received two sensitizing injections of *S. japonicum* eggs (A), and unsensitized control mice (B).

Delayed type hypersensitivity (DTH) ear assay

10 µl of ABCD eluate (approx. 160 egg equivalents) was injected into the left ear of five *S. japonicum* egg-sensitized C57BL/6 mice. Significant swelling in the left versus the right ear was detected using a microcaliper at 24 and 48 h. The mean change in ear thickness (±SEM) at these two time points in egg-sensitized mice was 100 µm ± 38 and 96 µm ± 44, respectively, and in unsensitized mice, 31 µm ± 21 and 32 µm ± 18, respectively. Injection of the same purified antigen into C57BL/6 and BALB/c mice infected with *L. major* did not elicit a cross-reactive DTH response.

Discussion

Numerous McAbs reacting with *S. japonicum* have been produced using spleen cells of BALB/c mice sensitized with egg or adult worm extracts of *S. japonicum* or with antigens of other parasites. Included in this battery are some McAbs described previously. Ten McAbs derived from the newer IM series of fusions that have not been reported were demonstrated to be reactive with *S. japonicum* egg antigens. These were classified into three groups, namely A, B and C by CRIAs. P.41–1–3 and SEF.85–5–3, two McAbs that were shown to cross inhibit in CRIAs, were classified as the D group. Using sera from several inbred strains of mice sensitized with C-McAb coupled to KLH, A, B and C-McAbs were shown to be idiotypically related through CRIA analysis. Interestingly, McAbs derived from the same set of fusions (the IM series) shared idiotypes and their target epitopes can be found on the same antigenic molecule. In contrast, the D-McAb, P.41–1–3, which is derived from another fusion, does not share this idiotype, although the target epitope can also be associated with the A, B and C-epitopes on the same egg molecule(s). The McAb P.41–1–3 was initially raised against *Paragonimus westermani* worm antigens (Cruise

et al., 1981b) and a McAb that reacts with *S. japonicum* egg antigens and also with *Paragonimus* and *Clonorchis* antigens has recently been reported by Miao et al. (1987).

The carbohydrate nature of A, B, C and D-epitopes was suggested by their sensitivity to mild periodate oxidation. Most McAbs that are specific for carbohydrate have been shown to be directed against non-reducing terminal sugars (Kannagi and Hakomori, 1986) and these are generally sensitive to periodate oxidation (Woodward et al., 1985). Whether A, B, C or D-epitopes contain non-reducing terminal sugars is not known. That antigens containing these epitopes are not precipitable by TCA adds support for the carbohydrate nature of these epitopes/antigens. This is also suggested by the 'smeary' appearance of the egg antigens recognized by these McAbs on Western blots, which is characteristic of carbohydrate-containing antigens. The *immunogenicity* of the D-epitope is severely affected by pronase treatment (unpublished observations); it might be associated with a protein moiety in part.

The relationship between the A, B, C and D-epitopes was further elucidated by a series of IRMAs and CRIAs. Two important variants of the IRMA were used, the two-step IRMA being more sensitive particularly for the detection of heterologous combinations of epitopes. The A, B, C and D-epitopes can be found in multiple homologous or heterologous combinations on one antigen molecule (or aggregate of antigens) (Fig. 1). The expression of A and D-epitopes seems to be developmentally regulated. The A-epitope is not found in immature egg and adult worm antigens. D-epitopes are found in much lower concentrations in immature egg antigens and, in the worm antigens, they are not combined with B and C-epitopes. A and D-epitopes are also species specific as both were not found in *S. mansoni* egg antigens, although sera from BALB/c infected with *S. mansoni* and therefore exposed to worm antigens as well, showed (minimal) inhibition of A-McAb in a CRIA.

Weiss and Strand (1985) described five McAbs that react with ConA-binding *S. mansoni* glycoproteins and that crossreact with cercarial and/or adult worm antigens. The stage and species specificity of the target epitopes was studied and four of the five can be found on egg glycoprotein antigens of *S. japonicum*. These antigens are very different from the glycoprotein egg antigens of *S. mansoni* as analyzed by two-dimensional electrophoresis. Some of these epitopes can be associated with one another as determined by CRIAs and by the similarities in appearance of the egg glycoprotein antigens immunoprecipitated by these McAbs and analyzed by two-dimensional electrophoresis. Like the A, B, C and D-epitopes, the target epitopes of these five McAbs are sensitive to periodate oxidation. They can also be found on glycolipid antigens of *S. mansoni* (Weiss et al., 1987). With regard to the A, B and C-epitopes, it is presently unknown whether they are epitopes on polysaccharide and/or glycoprotein and/or glycolipid antigens.

The results of T cell proliferation and DTH ear assay suggest that egg antigens containing the A, B, C and D-epitopes are immunopathologic. Granuloma formation in schistosomiasis japonica has been shown to be T cell mediated and is a classical DTH response (reviewed by Stavitsky, 1987). We have preliminary data that the ABCD eluate will sensitize mice for lung granuloma formation. However, considerable activity in recipients of the control WIC eluate suggests that contamination is a problem using immunoaffinity approaches and in vivo experiments involving hypersensitization. A possible biological function of the target epitopes of these McAbs

may be their ability to induce production of 'blocking' antibodies thus protecting the schistosomules (and adult worms) from immune attack. McAbs, or polyclonal antibodies from chronic mouse sera (Yi et al., 1986a,b; Dunne et al., 1987) that recognize carbohydrate cross-reactive epitopes between *S. mansoni* egg and schistosomular antigens have been shown to inhibit in vitro antibody-dependent cellular cytotoxicity (ADCC) against *S. mansoni* schistosomules. The occurrence of IgM blocking antibodies in human *S. mansoni* infections has been described (Khalife et al., 1986; Butterworth et al., 1987). The involvement of the McAbs described here (that are available on request) in inhibition of ADCC reactions has yet to be determined.

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References

Boros, D.L. and Warren, K.S. (1970) Delayed hypersensitivity-type granuloma formation and dermal reactions induced and elicited by a soluble factor isolated from *Schistosoma mansoni* eggs. J. Exp. Med. 132, 488–507.

Burnette, W.M. (1981) 'Western blotting': electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112, 195–203.

Butterworth, A.E., Bensted-Smith, R., Capron, A., Capron, M., Dalton, P.R., Dunne, D.W., Grzych, J.-M., Kariuki, H.C., Khalife, J., Koech, D., Mugambi, M., Ouma, J.H., Arap-Siongok, T.K. and Sturrock, R.F. (1987) Immunity in human schistosomiasis mansoni: prevention by blocking antibodies of the expression of immunity in young children. Parasitology 94, 281–300.

Carter, C.E. and Colley, D.G. (1981) *Schistosoma japonicum* soluble egg antigens: separation by Con A chromatography and immunoaffinity purification. Mol. Immunol. 18, 219–225.

Cruise, K.M., Mitchell, G.F., Garcia, E.G. and Anders, R.F. (1981a) Hybridoma antibody immunoassays for the detection of parasitic infection: further studies on a monoclonal antibody with immunodiagnostic potential for schistosomiasis japonica. Acta Trop. 38, 437–447.

Cruise, K.M., Mitchell, G.F., Tapales, F.P., Garcia, E.G. and Huang, S.R. (1981b) Murine hybridoma-derived antibodies producing circumoval precipitation (COP) reactions with eggs of *Schistosoma japonicum*. Aust. J. Exp. Biol. Med. Sci. 59, 503–515.

Cruise, K.M., Mitchell, G.F., Garcia, E.G., Tiu, W.U., Hocking, R.E. and Anders, R.F. (1983) Sj23, the target antigen in *Schistosoma japonicum* adult worms of an immunodiagnostic hybridoma antibody. Parasite Immunol. 5, 37–46.

DeIbarra, A.A.L., Howard, J.G. and Snary, D. (1982) Monoclonal antibodies to *Leishmania tropica major*: specificities and antigen location. Parasitology 85, 523–531.

Dunne, D.W., Bickle, Q.D., Butterworth, A.E. and Richardson, B.A. (1987) The blocking of human

antibody-dependent, eosinophil-mediated killing of *Schistosoma mansoni* schistosomula by monoclonal antibodies which cross-react with a polysaccharide-containing egg antigen. Parasitology 94, 269–280.

Forsyth, K.P., Mitchell, G.F. and Copeman, D.B. (1984) *Onchocerca gibsoni*: increase of circulating egg antigen with chemotherapy in bovines. Exp. Parasitol. 58, 41–55.

Forsyth, K.P., Spark, R., Kazura, J., Brown, G.V., Peters, P., Heywood, P., Dissanayake, S. and Mitchell, G.F. (1985) A monoclonal antibody-based immunoradiometric assay for detection of circulating antigen in *Bancroftian filariasis*. J. Immunol. 134, 1172–1177.

Garcia, E.G., Tapales, F.P., Valdez, C.A., Mitchell, G.F. and Tiu, W.U. (1981) Attempts to standardize the circumoval precipitin test (COPT) for schistosomiasis japonica. Southeast Asian J. Trop. Med. Pub. Hlth. 12, 384–394.

Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89, 114–123.

Handman, E., Greenblatt, C.L. and Goding, J.W. (1984) An amphipathic sulphated glycoconjugate of *Leishmania*: characterization with monoclonal antibodies. EMBO J. 3, 2301–2306.

Hillyer, G.V. and Pelley, R.P. (1980) The major serological antigen (MSA_1) from *Schistosoma mansoni* eggs is a circumoval precipitinogen. Am. J. Trop. Med. Hyg. 29, 582–585.

Johnson, D.A., Gautch, J.W., Sportman, J.R. and Elder, J.H. (1984) Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Techn. 1, 3–8.

Kannagi, R. and Hakomori, S. (1986) Monoclonal antibodies directed to carbohydrate antigens. In: D.M. Weir, L.A. Herzenberg, C. Blackwell, L.A. Herzenberg (Eds.), Handbook of Experimental Immunology, Blackwell, Oxford, pp. 117.1–117.20.

Khalife, J., Capron, M., Capron, A., Grzych, J.-M., Butterworth, A.E., Dunne, D.W. and Ouma, J.H. (1986) Immunity in human schistosomiasis mansoni. Regulation of protective immune mechanisms by IgM blocking antibodies. J. Exp. Med 164, 1626–1640.

Kowal, R. and Parsons, R.G. (1980) Stabilization of proteins immobilized on Sepharose from leakage by gluteraldehyde cross linking. Anal. Biochem. 102, 72–76.

Lewert, R.M., Yogore, M.G. Jr., Martin, W.R. and Blas, B.L. (1980) Schistosomiasis japonica in Barrio San Antonio, Basey, Samar in the Philippines. IV. 'Atypical' precipitates in the circumoval precipitin test – a reaction of recent infection. Am. J. Trop. Med. Hyg. 29, 431–434.

Long, G.W., Lewert, R.W. and Pelley, R.P. (1981a) Characterization of the glycoprotein antigens which mediate the *Schistosoma japonicum* circumoval precipitin reaction. Infect. Immun. 34, 389–396.

Long, G.W., Lewert, R.M. and Pelley, R.P. (1981b) Fractionation of *Schistosoma japonicum* soluble egg antigen glycoproteins by hydrophobic interaction chromatography. Infect. Immun. 34, 397–406.

Miao, D.J., Zhu, R.S., Zhang, S.H., Zhou, R., Huang, Q.H., Yan, Z.Z. and Pan, X.Q. (1987) Cell lines of hybridoma secreting monoclonal antibodies against *Schistosoma japonicum* egg antigens. Chinese J. Parasitol. Parasitic Dis. 5, 9–12.

Mitchell, G.F., Cruise, K.M., Garcia, E.G. and Anders, R.F. (1981a) Hybridoma derived antibody with immunodiagnostic potential for schistosomiasis japonica. Proc. Natl. Acad. Sci. U.S.A. 78, 3165–3169.

Mitchell, G.F., Garcia, E.G., Anders, R.F., Valdez, C.A., Tapales, F.P. and Cruise, K.M. (1981b) *Schistosoma japonicum*: infection characteristics in mice of various strains and a difference in the response to eggs. Int. J. Parasitol. 11, 267–276.

Mitchell, G.F., Cruise, K.M., Garcia, E.G., Vadas, M.A. and Munoz, J.J. (1983a) Attempts to modify lung granulomatous responses to *Schistosoma japonicum* eggs in low and high responder mouse strains. Aust. J. Exp. Biol. Med. Sci. 61, 411–424.

Mitchell, G.F., Garcia, E.G., Cruise, K.M., Tiu, W.U. and Hocking, R.E. (1982) Lung granulomatous hypersensitivity to eggs of *Schistosoma japonicum* in mice analysed by a radioisotopic assay and effects of hybridoma (idiotype) sensitization. Aust. J. Exp. Biol. Med. Sci. 60, 401–416.

Mitchell, G.F., Garcia, E.G., and Cruise, K.M. (1983b) Competitive radioimmunoassays using hybridoma and anti-idiotype antibodies in identification of antibody responses to, and antigens of, *Schistosoma japonicum*. Aust. J. Exp. Biol. Med. Sci 61, 27–36.

Mitchell, G.F., Premier, R.R., Garcia, E.G., Hurrell, J.G.R., Chandler, H.M., Cruise, K.M., Tapales, F.P. and Tiu, W.U. (1983c) Hybridoma antibody-based competitive ELISA in *Schistosoma japonicum* infection. Am. J. Trop. Med. Hyg. 32, 114–117.

Norden, A.P. and Strand, M. (1984) *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*: identification of genus- and species-specific antigenic egg glycoproteins. Exp. Parasitol. 58, 333–344.

Pesigan, T.P., Farooq, M., Hairston, N.G., Jauregui, J.J., Garcia, E.G., Santos, A.T., Santos, B.C. and

Besa, A.A. (1958) Studies on *Schistosoma japonicum* infection in the Philippines. I. General consideration and epidemiology. Bull. WHO 18, 345–455.

Seppala, I.J.T. and Eichmann, K. (1979) Isolation and characterization of isogeneic anti-idiotypic antibodies to BALB/c myeloma S117: lack of reactivity with major idiotypic determinants. Eur. J. Immunol. 9, 243–250.

Sidner, R.A., Carter, C.E. and Colley, D.G. (1987) Modulation of *Schistosoma japonicum* pulmonary egg granulomas with monoclonal antibodies. Am. J. Trop. Med. Hyg. 36, 361–370.

Smithers, S.R. (1960) The isolation of viable schistosoma eggs by a digestion technique. Trans. R. Soc. Trop. Med. Hyg. 54, 68–70.

Stavitsky, A.B. (1987) Immune regulation in schistosomiasis japonica. Immunol. Today 8, 228–233.

Tracy, J.W. and Mahmoud, A.A.F. (1982) Isolation of *Schistosoma japonicum* egg glycoprotein antigens which sensitize mice to lung granuloma formation and elicit an immediate hypersensitivity response. Am. J. Trop. Med. Hyg. 31, 1201–1212.

Tsai, C.M. and Frasch, C.E. (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119, 115–119.

Tsang, V.C.W. (1982) New methods of purifying and identifying parasitic diagnostic antigens: a review of the systematic and quantitative approach with the *Schistosoma* model. Vet. Parasitol. 10, 171–179.

Vogel, H. (1942) On the development, length of life, and death of the eggs of *Bilharzia japonica* in the tissue of the host. Deutsch. Trop. Z. 46, 57–69.

Weiss, J.B. and Strand, M. (1985) Characterization of developmentally regulated epitope of *Schistosoma mansoni* egg glycoprotein antigens. J. Immunol. 135, 1421–1429.

Weiss, J.B., Aronstein, W.S. and Strand, M. (1987) *Schistosoma mansoni*: stimulation of artificial granuloma formation in vivo by carbohydrate determinants. Exp. Parasitol. 64, 228–236.

Woodward, M.P., Young, Jr., W.W. and Blood, R.A. (1985) Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Methods 78, 143–153.

Yi, X.Y., Omer-Ali, P., Kelly, C., Simpson, A.J.G. and Smithers, S.R. (1986a) IgM antibodies recognizing carbohydrate epitopes shared between schistosomula and miracidia of *Schistosoma mansoni* that block in vitro killing. J. Immunol. 137, 3946–3954.

Yi, X.Y., Simpson, A.J.G., de Rossi, R. and Smithers, S.R. (1986b) The presence of antibody in mice chronically infected with *Schistosoma mansoni* which blocks in vitro killing of schistosomula. J. Immunol. 137, 3955–3958.