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Anti-schistosome monoclonal antibodies of different isotypes – correlation with cytotoxicity

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Five monoclonal antibodies specific towards *Schistosoma mansoni* antigens were prepared by fusion of spleen cells of infected and immunized mouse with the murine myeloma NS-1 cells. Three of the five antibodies belonged to the IgG_1 class, one was an IgM and the fifth one was an IgE. The IgE monoclonl antibody designated 54.10, induced antigen-specific degranulation of rat basophilic cell line, a property which served as the basis for the screening assay. Its biological function was demonstrated by a specific macrophage activation that led to killing of schistosomula; no such killing was obtained with anti-schistosome antibodies of other classes or with IgE of different antigenic specificity. The second monoclonal antibody of biological significance was an IgG_1, designated 27.21 which is reactive in the immunofluorescence staining of surface antigens on intact schistosomula. All three monoclonal antibodies that belonged to the IgG_1 class were effective in mediating killing of schistosomula by complement, with the highest effect exerted by 27.21. It is thus apparent that the 27.21 monoclonal antibody is directed against a densely distributed surface antigen on the schistosomula membrane which is possibly involved in the protective immunity. Preliminary data showed that immunoprecipitation with the 27.21 antibodies results in the isolation of three major protein bands, of 60 kd, 50 kd, 19 kd, respectively.
Key words: cytotoxicity/IgE-producing hybridoma/immunofluorescence/monoclonal antibodies/*Schistosoma*

Introduction

The processes involved in the immune responses against parasites are very complex. This stems both from antigenic variations during the life cycle and the multitude of antigenic components comprising the parasitic organism, as well as from the very mechanism by which the parasites evade the immune system of the host. In such an intricate system, the monoclonal antibodies offer great advantages, for the following reasons: (1) even though the antigens involved are many and complex, each monoclonal antibody will be of absolute narrowly defined specificity; (2) monoclonal antibodies could be screened for specificity towards a given stage in the development of the parasite, so as to detect stage-specific antigens or antigens that are shared by different forms of the parasite; (3) availability of monoclonal antibodies could lead to the isolation of defined parasite antigens, hopefully antigens of protective potential. Indeed, several reports on the use of monoclonal antibodies in several parasite systems have appeared during the last few years (Potocnjak *et al.*, 1980; Parkhouse *et al.*, 1981; Mitchell *et al.*, 1981; Butterworth *et al.*, 1982), culminating in the use of anti-sporozoite monoclonal antibodies and its monoclonal anti-idiotype for a specific diagnostic test for malaria (Potocnjak *et al.*, 1982).

There are several recent reports of monoclonal antibodies against *Schistosoma mansoni*. Verwaerde *et al.* (1979), described the preparation of monoclonal antibodies in rats against the *Schistosoma* larvae, whereas Butterworth *et al.* (1982) reported on the preparation of mouse IgG_1 and IgM monoclonal antibodies against schistosome surface antigens, which had no detectable biological activity, but allowed the isolation of a 24 000-dalton protein antigen.

We have reported on a procedure for immunization against *S. mansoni* which led to relatively efficient protection parallelled by high level of schistosome-specific IgE antibodies (Horowitz *et al.*, 1982). A cause and effect relationship between these two findings is possible, since the presence of a high level of IgE antibodies in cases of infection with *Schistosoma* (Sadun and Gore, 1970; Dessaint *et al.*, 1975; Rouseaux-Prevost *et al.*, 1978) or other helminthic diseases (Sadun, 1972; Oglivie and Jones, 1973; Jarret and Bazin, 1974; Ishizaka *et al.*, 1976; Musoke *et al.*, 1978), has been established. It was therefore envisaged that if spleens of mice immunized in this fashion are used in the fusion experiments, and if adequate assays for screening are employed, specific anti-schistosome antibodies of different classes, including IgE, could be selected and such antibodies might possess biological functions.

Here we report the preparation of five monoclonal antibodies of IgG, IgM and IgE classes, with high binding capacity to schistosome antigens. Furthermore, one of these antibodies belonging to the IgG_1 class, as well as the IgE monoclonal antibodies, mediated complement- or macrophage-dependent damage to the schistosomula *in vitro*.

Results

Preparation of hybridomas and characterization of the monoclonal antibodies

Three fusion experiments were carried out using spleens of mice sensitized by different procedures. For the first fusion, $B6D2F_1$ mice were immunized with 2 μg cercarial antigen (CA) in alum, followed by three boosters with similar amount of the antigen; this is the procedure used previously for protective immunization. For the second fusion DBA/2 mice were twice infected with 50 cercaria; for the third and most successful fusion mice were first twice infected by injection of 50 cercaria and then immunized with 1 μg CA in alum. In all three fusions an additional booster with 1 μg CA in alum was administered 4 days before fusion.

The most positive results were obtained from the third fusion experiment. Approximately 10% of the cultures exhibited binding to the CA in radioimmunoassay (RIA) but only one of them was IgE-positive in the rat basophilic leukemia cells (RBL) degranulation assay (Taurog, 1977). The cultures were propagated *in vitro* and the five highest antibody-producing hybridomas, including the one reacting positively in the RBL degranulation assay, were further cloned by the soft agar cloning technique. The cloned lines were immunochemically characterized, and propagated *in vivo* as

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Table I. Isotypes of monoclonal antibodies by RIA

Clone number	RIA with goat antiserum against					Rabbit α-m IgE	RBL degranulation assay	McAb isotype
	IgA	IgM	IgG_1	IgG_{2a}	IgG_{2b}			
11.15	3715	41 043	2773	1492	2650	2473	1698	IgM
26.12	1333	2913	15 387	2413	1882	2975	1430	IgG_1
27.21	1915	2742	17 977	2202	3687	2573	3584	IgG_1
54.10	1385	3901	2875	1345	2154	15 656	41 074	IgE
58.7	1762	3827	16 025	1939	3293	11 091	1506	IgG_1

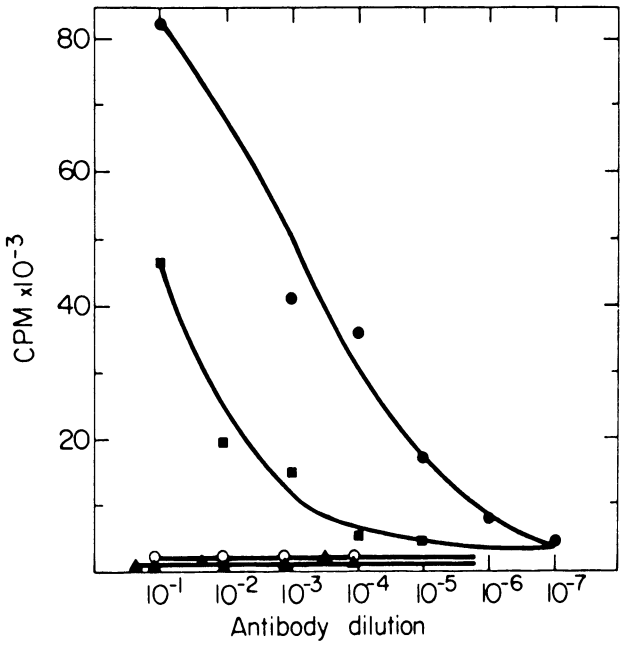

Fig. 1. Monoclonal IgE antiboies against *S. mansoni* measured by [^{3}H]serotonin release from RBL cells. CA with (●) 54.10 ascitic fluid (IgE), with (■) 54.10 hybridoma supernatant (IgE), with (▲) 27.21 ascitic fluid (IgG_1), and (○) 54.10 ascitic fluid with non-relevant antigen (DNP-BSA).

ascites in mice.

Isotype determination was carried out by gel diffusion and by RIA. In gel diffusion experiments, one hybridoma product (11.15) reacted with anti-mouse IgM serum; one hybridoma product (27.21) reacted with anti-mouse IgG_1 serum, and a third (54.10) reacted with anti-mouse IgE. The two other hybridoma products (26.12 and 58.7) did not give visible precipitin bands with any of the anti-mouse antisera. None of the hybridomas responded with anti-mouse IgA serum.

Quantitative evaluation of the isotype determination was obtained by the RIA of the respective ascitic fluids using the sonicated cercaria as antigen. According to their reactivity with the specific antisera, the five monoclonal antibodies belonged, respectively, to the IgM, IgG_1 and IgE classes (Table I). The IgG_1 mcAb 58.7 bound also with anti-IgE; however, when tested for the presence of IgE antibodies by the specific RBL degranulation assay (the column before last in Table I), only monoclonal antibody 54.10 gave a positive response. A similar pattern of reactivity was obtained when a sonicate of schistosomula were used as an antigen, and a lower degree of reactivity was observed with a sonicate of adult worms.

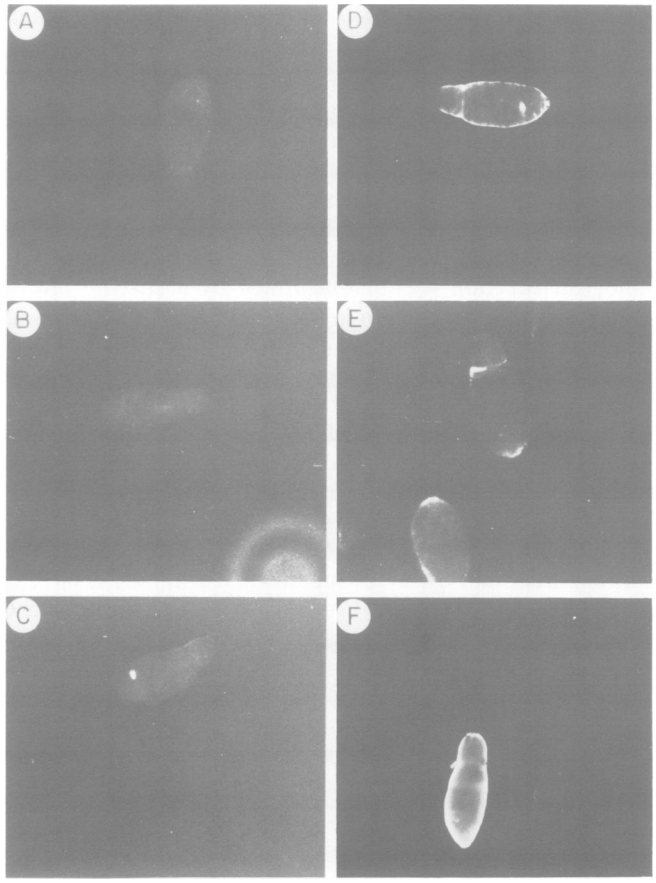

Fig. 2. Fluorescence staining of schistosomula with monoclonal antibodies. **(A)** without antibodies; **(B)** with normal mouse serum; **(C)** with irrelevant monoclonal antibodies anti-T-G-AL; **(D)** with IgG_1 anti-*S. mansoni* (27.21 mcAb) diluted 1:2; **(E)** 27.21 diluted 1:5; **(F)** serum from infected mice.

The level of IgE antibodies in the 54.10 hybridoma products was determined by a quantitative assay of the release of [^{3}H]serotonin from RBL cells. Figure 1 illustrates the specificity and titer of this product. In this assay which follows the biological function of the IgE antibodies, the ascitic fluid of 54.10 was capable of causing degranulation of basophils up to a dilution of 10^{-6} in the presence of CA sonicate, but not upon addition of an irrelevant antigen. None of the other hybridoma products reacted in this assay, as shown in Figure 1 by the results with the monoclonal IgG_1 27.21.

Binding of monoclonal antibodies to intact schistosomula

The results reported hitherto demonstrate the reactivity of the monoclonal antibodies with the CA sonicate, which contains both soluble antigens and membranal components of both cercariae and schistosomula. The following experiment

Table II. Complement-dependent cytotoxic effect of mcAb on *S. mansoni*. A typical experiment

Antibody[a]	With complement			With inactivated complement			Net percent dead
	No. schistosomula		Percent dead	No. schistosomula		Percent dead	
	Dead	Live		Dead	Live		
McAb:[b]							
11.15	53	117	31	18	80	18	13
26.12	48	65	42	9	82	10	32
27.21	140	62	69	15	91	14	55
54.10	54	81	40	17	72	19	21
58.7	80	76	51	11	67	14	37
n.r. IgG[c]	64	99	39	21	81	20	19
Control sera:							
Normal	26	68	27	17	77	18	9
Infected	126	29	81	10	63	16	65

[a]Antibody dilution was 1/10 in DSM.
[b]Sera and ascitic fluids (except the IgE) were decomplemented prior to test.
[c]Non-relevant ascitic fluid anti-(T,G)-A–L

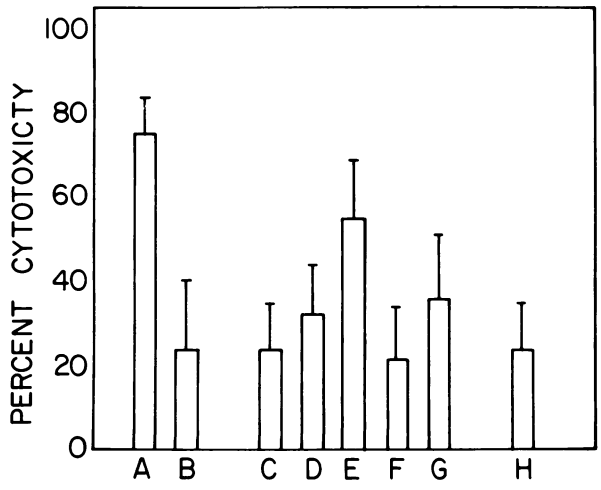

Fig. 3. Complement-mediated cytotoxic effect of monoclonal antibodies on *S. mansoni*. **(A)** serum from infected mice; **(B)** normal mouse serum; **(C)** mcAb IgM (11.15); **(D)** mcAb IgG_1 (26.12); **(E)** mcAb IgG_1 (27.21); **(F)** mcAb IgE (54.10); **(G)** mcAb IgG_1 (58.7); **(H)** mcAb (T,G)-A–L (IgG).

demonstrates their binding capacity to intact schistosomula, as manifested by immunofluorescence (Figure 2). The staining reagent, fluorescein isothiocyanate (FITC)-labeled goat anti-mouse did not give any fluorescence upon addition to the schistosomula as such (A) or after interaction with normal mouse serum (B) or with irrelevant ascitic fluid (C). A strong staining was obtained, as expected, upon interaction of the schistosomula with serum of infected mouse (F). Of all five anti-schistosome mcAb the only one reactive in this assay was 27.21 (D), which gave very significant and consistent staining also at a dilution of 1:5 (E). With the other four anti-CA ascitic fluids, particularly with that of 54.10, an occasional staining of an individual schistosomulum was observed. This partial binding was confirmed by a more sensitive radiolabeling experiment using [^{125}I]Protein A.

Complement-dependent cytotoxicity

The capacity of all five anti-schistosome monoclonal antibodies to mediate killing of schistosomula by complement is demonstrated in Table II and Figure 3. The highest killing effect was obtained in the presence of the monoclonal IgG_1 27.21 (Figure 3E). Table II, which gives the data of a typical experiment, demonstrates that in the absence of active complement, between 10 and 20% non-specific killing was observed in all cases. The net complement-dependent toxicity, which is the difference between the killing in the presence of fresh and inactivated complement, was 55% with the IgG_1 monoclonal antibody 27.21, close to the positive control value obtained with the serum of infected mice. The other monoclonal antibodies gave lower cytotoxicity levels. Figure 3 shows the average results obtained from 12 different experiments. The IgE monoclonal antibodies showed no cytotoxic activity in this assay, probably because it does not bind complement; on the other hand monoclonal antibody 58.7 which belongs to the IgG_1 class, also showed a considerable cytotoxic effect (Figure 3G).

Macrophage-dependent cytotoxicity

To assess whether the IgE monoclonal antibody is active against the living schistosomula, we looked for its capacity to activate macrophages into cytotoxic effector cells. The results of three experiments are given in Table III. As shown, the IgE anti-schistosome monoclonal antibodies 54.10 indeed resulted in macrophage-dependent toxicity. Irrelevant IgE monoclonal antibodies (anti-DNP) exerted a low toxic effect whereas IgG_1 monoclonal antibodies, either anti-schistosome or with irrelevant specificity, showed no effect.

Identification of antigen

An attempt to identify an antigen that might be relevant to the complement-mediated toxicity was made by immunoprecipitation with the IgG_1 monoclonal antibodies 27.21. The results of a preliminary experiment are presented in Figure 4. As shown, under denaturing conditions of SDS-polyacrylamide gel electrophoresis, three major radioactive bands of 19 kd, 50 kd and 60 kd, respectively, and a minor band of 23 000-dalton were precipitated with the 27.21 antibodies, but not with an irrelevant mcAb (lane 4), or without addition of antibodies (lane 3). The quantity of the antigen was too low to be detected by the protein stain, and they are, therefore, visible only in the autoradiogram.

Discussion

The results presented above show that two of the monoclonal antibodies prepared in this study possess clear-cut biological activity towards the schistosome larvae.

Table III. Apparent killing by macrophages activated by mcAb

Antibody[a]	Percent dead schistosomula (mean of each experiment)									Mean ± S.E.[e]
	Experiment I (duplicates)			Experiment II (triplicates)			Experiment III (quadruplicates)			
	+ MØ	− MØ	Net	+ MØ	− MØ	Net	+ MØ	− MØ	Net	
McAb[b]										
54.10	53	18	35	47	12.2	34.8	83.3	29.3	54.0	40.7 ± 5.1
27.21	38	30	8	14.9	8.4	6.5	40.5	34.3	6.2	7.4 ± 7.1
58.7	33	27	6	25.4	18.6	6.8	31.9	29.9	3.0	4.7 ± 2.8
n.r. IgE[c]	39	27	12	44.3	21.2	23.1	38.5	29.9	8.6	14.4 ± 3.4
n.r. IgG[d]	N.D.	N.D.	N.D.	14.8	15.2	0	33.1	30.0	3.1	1.5 ± 3.9
Control sera										
Normal	62	57	5	44	45.7	0	35.7	26.2	9.5	7.0 ± 4.4
Infected	59	25	34	66	21.5	44.5	56.8	30.8	26.0	34.0 ± 3.3
No antibodies	14	18	0	17	19.8	0	N.D.	N.D.	N.D.	−3.8 ± 1.5

[a]Antibody dilution was 1/10 in EM/FCS 10%.
[b]Not decomplementated fresh ascitic fluids.
[c]Non-relevant IgE with anti-DNP specificity.
[d]Non-relevant ascitic fluid anti-(T,G)-A–L
[e]Mean values calculated for all the individual wells in the three different experiments.

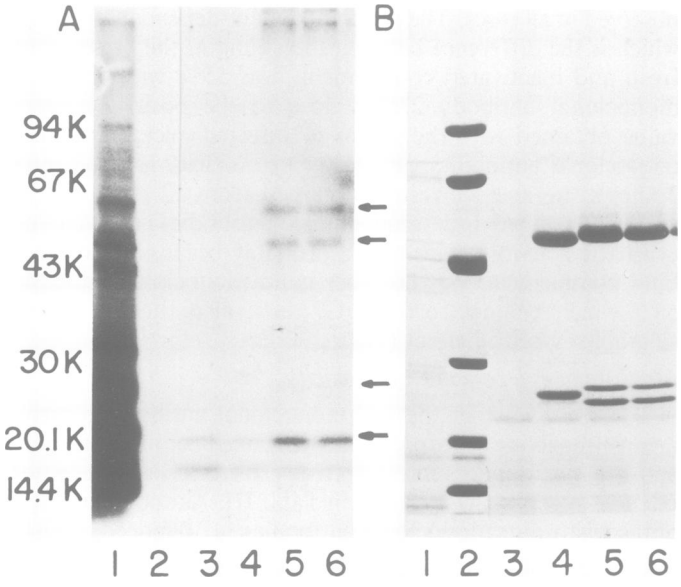

Fig. 4. SDS-polyacrylamide gel electrophoresis of immunoprecipitation of ^{125}I-labeled CA with mcAb 27.21. **(A)** Autoradiogram. **(B)** Protein stain. **Lane 1**: CA extract adsorbed on *S. aureus* and used as the antigen for the IP; **lane 2**: unlabeled markers, **lanes 3–6** supernates from immunoprecipitates of the CA with: no antibodies **(3)**; anti(T,G)-A–L mcAb **(4)**; 27.21 mcAb 1:20 **(5)**; 27.21 mcAb 1:40 **(6)**. The protein stain indicates the positions of light and heavy chains of the antibodies in the immunoprecipitate. The radioactivity bands designates the polypeptide chains of the parasite's antigen(s), the quantity of which is too low to be detected by the protein stain.

The immunogens used for provoking the antibodies consisted of both the adult schistosomes (a consequence of the infection process) and the larval material (sonicated cercariae). The immunization procedure was the one previously reported (Horowitz *et al.*, 1982) to elicit high levels of IgE. In this manner the induction of antibodies against a wide range of antigenic specificity and of different classes was ensured. The rationale for that is the uncertainty about the antigen(s) which are of relevant function, whether they are stage specific or shared by adults and larvae, whether they are surface antigens or those secreted by one of the parasite forms.

The screening of the antibody-producing cultures was according to their binding capacity to the schistosome antigens. However, the screening for the IgE producer was feasible only because a sensitive and specific functional assay for IgE antibodies was available (Taurog *et al.*, 1977), based on the degranulation of basophils by the antibodies in the presence of the specific antigen. This assay was previously adapted in our laboratory for the detection of anti-schistosome IgE (Horowitz *et al.*, 1982). The hybridoma product 54.10 obtained in this study is one of a few monoclonal antibodies of the IgE class reported so far. The special interest in it within the context of the present work stems from the notion that antibodies of the IgE class may be involved in protective immunity against schistosomiasis, probably by mediating immune damage by macrophages (Capron *et al.*, 1975), or eosinophils (Capron *et al.*, 1981).

The functional properties of those IgE monoclonal antibodies was evaluated by two criteria: firstly, their capacity to bind to intact parasites – immunofluorescence showed occasional staining, and radiolabeling with Protein A indicated partial binding; secondly, their capacity to mediate immune damage. As expected, we did not observe complement-dependent cytotoxicity (Figure 3F), probably due to the lack of complement binding sites on IgE antibodies. In contrast, the antibodies were capable of inducing macrophage activation (Table III) in a specific manner. The effect observed was restricted to antibodies of the IgE class with the relevant antigenic specificity. These data are in accordance with the findings by Capron *et al.* (1982) on the capacity of IgE antibodies in sera of infected mice and rats to induce killing of schistosomula by macrophages.

The second monoclonal antibody exerting biological function is of the IgG_1 class (designated 27.21). These antibodies showed clear-cut binding to surface antigens on the intact schistosomula in an immunofluorescence assay (Figures 2D and 2E). More importantly, their interaction with the parasite resulted in high complement dependent killing effect on live schistosomula (Figure 3E and Table II). It is of interest that the two other hybridoma products that are effective in this cytotoxicity assay, but to a lesser extent than 27.21, are also of the IgG_1 class.

Both immunofluorescence staining and the complement-

induced cytotoxicity for rupture of cell membrane by complement, are processes that depend on the presence of appropriate receptors on the membrane, and consequently can take place only if the respective binding moiety is present in fair amount on the cell surface. It is thus apparent that the 27.21 antibodies are directed towards an abundant surface antigen of the schistosomula. The same monoclonal antibodies react in the RIA also with sonicates of the adult worms, and thus could point to the existence of shared antigens. However, the location of such antigens in the different stages has not yet been determined.

A corroboration for this assumption is the isolation of distinct protein components from a soluble cercarial extract by immunoprecipitation with the 27.21 monoclonal antibodies. Further characterization of these proteins is needed to determine whether they are of any significance in conferring immunity against schistosomiasis.

Materials and methods

Parasite and antigen

A Pueto-Rican strain of *S. mansoni* is maintained in our laboratory. Cercarial antigen (CA) was prepared by sonication as previously described (Horowitz *et al.*, 1982). Schistosomula, mechanically transformed, were prepared as described by Levi-Schaffer and Smolarsky (1982).

Mice

Mice of the inbred strains DBA/2; C57BL/6J; (C57BL/6JxDBA/2)F_1 (B6D2F_1); (BALB/cxDBA/2)F_1 (CD2F1), the outbred ICR and (C3H/ebx-C57BL/6J)F_1 were obtained from the Animal Breeding Center, Weizmann Institute, Rehovot.

Immunization

Female DBA/2 mice 8–10 weeks of age were infected twice with 50 cercaria 9 weeks apart, and then immunized with 1 μg CA adsorbed on alum. The antisera were tested for titer of anti-schistosome IgG and IgE antibodies (as described below) and the spleens of the mice with the highest titer were taken for the fusion.

Hybridization

Four days prior to the fusion the mice were boosted with 1 μg CA in alum, and spleen cells from the infected-immunized mice were fused with NS-1 myeloma cells at a 5:1 ratio using 41% (w/v) PEG-1500 as a fusing agent following the procedure described before (Eshhar *et al.*, 1980; Galfré *et al.*, 1977). After fusion the cells were divided into 500 wells in microtiter plates (Falcon Plastics, Oxnard, CA), and two days later the cells were subjected to the selective HAT medium.

Screening of specific anti-schistosome hybridomas

Hybrid cells growing in HAT selective medium were selected for secretion of anti-schistosome Ig by two assays: (1) solid phase RIA using CA sonicate as antigen and performed as described previously (Horowitz *et al.*, 1982). (2) basophils degranulation assay – using RBL of the secreting sub-line 2H3 which were kindly provided by H.Metzger (NIH, Bethesda, MD). The assay for antibodies of the IgE isotype (Taurog, 1977) was performed for detection of anti-schistosome antibodies of the IgE isotype as described previously (Horowitz *et al.*, 1982). The hybrid cultures positive in the screening assays were cloned by the soft agar technique. Cells from isolated clones were grown in culture and also as ascites in CD2F_1 mice.

Determination of isotypes

Supernatants of hybridoma cultures were tested in double immunodiffusion in agar. Ascitic fluids of clones were tested in RIA using goat anti-mouse isotypes sera (Meloy, Springfield, VA) and purified ^{125}I-labelled rabbit anti-goat IgG. RIA for IgE monoclonal antibodies was performed with purified rabbit anti-mouse IgE and ^{125}I-labelled goat anti-rabbit IgG-reagents. All the purified reagents were prepared by ion-exchange chromatography followed by affinity chromatography on the relevant immunoglobulins and labelling with ^{125}I was carried out by chloramine T methods as described previously (Horowitz *et al.*, 1982).

Immunofluorescence staining

3 h schistosomula were reacted for 30 min at 37°C with the various mcAb containing ascitic fluids, at a 1:2 dilution, and with different sera to be tested, undiluted. After three washings, FITC-goat anti-mouse IgG (Miles-Yeda, Rehovot), was added (1:20 dilution) and incubation proceeded for 20 min at 0°C. After 1 h fixation in 1.0 ml 3% paraformaldehyde the worms were observed and photographed in a fluorescent microscope (Zeiss, photomicroscope III on a Kodak Trix film) using the same exposure for all the samples. In the case of IgE antibodies the specific reagent was rabbit anit-mouse IgE, followed by FITC-goat anti-rabbit IgG (Miles-Yeda, Rehovot).

Complement-dependent cytotoxicity

The ascitic fluids and the sera (except the IgE) were decomplemented (56°C for 30 min), centrifuged to remove aggregates and rendered sterile either by filtration (0.45 μm filter, Gelman) or by continuous sonication. 3 h schistosomula, 150–200 in 0.1 ml, were incubated with the antibodies at various dilutions for 30 min at 37°C. Complement, diluted 1:2.5 (0.1 ml of either fresh or heat inactivated guinea pig serum) was added. The live, dead and damaged worms were counted microscopically after 18 h incubation at 37°C.

Macrophages-dependent cytotoxicity

Peritoneal macrophages (MØ) were prepared from peritoneal exudate cells (PEC) as described elsewhere (Tzehoval, 1978). The assay was performed in microtiter plates (Falcon, Oxnard, CA). The MØ (10^5 cells/well) were incubated with the antibody samples at a 1:10 dilution for 6 h, followed by addition of 3 h schistosomula (50–60 to each well). The percentage of dead schistosomules was determined microscopically after 18 h incubation at 37°C.

Immunoprecipitation

The ^{125}I-labelled antigen consisted of a one to one mixture of sonicated cercaria (CA) labelled by Bolton and Hunter reagent, and cercarial sonicate labelled by chloramine T method. This labelled mixture (600 μg protein) was again sonicated, solubilized with 1% Nonidet P-40, ultracentrifuged (105 000 *g*) and mixed with similarly prepared extract of unlabelled CA (25 mg protein). Immunoprecipitation was performed basically according to Kessler (1975) with slight modifications. The antigen was preadsorbed on intact *Staphylococcus* A and then reacted with various amounts of the monoclonal antibodies. The precipitates obtained after addition of fixed *Staphylococcus* A were analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and by autoradiography.

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