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IDENTIFICATION OF SNAILS INFECTED WITH SCHISTOSOMA MANSONI BY THE USE OF MONOCLONAL ANTIBODIES AND DNA PROBES

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Executive Summary: Schistosomiasis, a water-borne disease transmitted by freshwater snails afflicts more than 200 million people globally. It is a lingering public health problem in many developing countries because chemotherapy does not prevent reinfection, and because the vicious circle of human-water-snail interactions that leads to transmission still prevails in these countries.

Determination of schistosome infections in humans and snails is the hallmark of epidemiological surveys, but so far snail infection rates cannot be accurately determined. Firstly,because routine tests only examine whether the snails shed infective larvae-cercaria, thus leaving out infected snails in which infection is not yet patent. Secondly, because cercarial shedding tests do not provide differential identification of human schistosomes larvae from animal schistosomes' larvae which may develop in the same snail species.

The present project was aimed at identifying infected snails by detecting schistosome-derived molecules (antigens, DNA) rather than the parasites themselves. For this purpose, we initially developed monoclonal antibodies (Mab) to Schistosoma mansoni and used them in a simple test system for detecting hitherto undefined schistosomal antigens in the hemolymph of infected snails. The test made it possible to differentially identify S. mansoni-infected snails from uninfected ones and from snails infected with other tromatodes. Both laboratory studies in in Jerusalem and field studies in Kenya proved

the high degree of sensitivity and specificity of the test and its ability to detect snails even before they shed cercariae.

DNA-DNA hybridization was also employed for detecting infected snails. For this purpose a simple procedure for preparing snails for DNA hybridization tests was developed, and highly repeated, tandemly arranged DNA sequences representing at least 12% of the S. mansoni genome were cloned in bacteria and used as probes. These probes proved to be species specific and capable of detecting S. mansoni infection in snails with high sensitivity.

The results of this project clearly demonstrate the feasibility of our approach and open the way for development of similar probes for other schistosome species. They justify further research and development and extended field trial in search of cost effective detection procedures.

This project helped the transfer of hybridoma technology as well as modern concepts and approaches of diagnostic biotechnology to the collaborating Kenyan laboratory.

Research Objectives. The overall aim of the present project was to prepare and apply Mab and DNA probes for the differential identification of snails infected with S. mansoni. None of these means of molecular identification of schistosome infection in snails existed when the project was started. Snail infection was and still is routinely monitored by examining the capacity of the snails to shed carcariae. Prepatent infections in the snails are not tested routinely at all although they may constitute a significant proportion of an infected snail populations in active transmission sites. Microscopical examination of crushed snails for presence of early larval stages or serial shedding tests over several weeks after transfer of the snails to the laboratory were carried out experimentally for detecting prepatent inferctions in snails. These approaches, however, are not considered suitable for routine testing because they are cumbersome and/or time consuming, and/or inaccurate.

More accurate determination of snail infection rates (including prepatent infections) was considered important because it should help more accurate application of mathematical models of transmission. Also, upscaling of transmission monitoring was obviated since mass control measures, particularly mass chemotherapy, were on the increase when this project was planned. Furthermore, rapid developments in schistosomiasis vaccine research also required a balancing development of suitable technologies for mass monitoring. It was argued that identification of active transmission sites by community surveys and studies on human

behaviour is expensive and often results in a decline in community participation. Snail populations, on the other hand, can be sampled more frequently and should allow more rapid detection of resurgent transmission after a mass control campaign.

The use of antibodies for detecting schistosomal antigens in infected snails was one of the approaches taken by us. The feasibility of this approach depended on the availability of antibodies that can clearly differentiate between schistosomal and snail antigens. We initially prepared polyclonal antibodies by immunizing rabbits with extracts of adult worms and cercariae. These antibodies exhibited stronger binding to infected snails extracts than to extracts of normal uninfected snails, but there was still a very high degree of cross reactivity. The widespread antigenic cross reactivity, between schistosomes and snails was well known (1-9) and since our polyclonal antibodies could not provide sufficient discrimination between infected and normal snails this work was discontinued, and a search for suitable monoclonal antibodies took place. Monoclonal antibodies that can differentially bind to schistosomal antigens in snails extracts have not been previously desribed but presence of schistosome-specific antigens in infected Biomphalaria glabrata was demonstrated in a single publication (10) by employing suitably absorbed infected mouse serum. Although the use of monoclonal antibodies for detecting parasites (e.g. malaria) in vectors (mosquitoes) is being applied for large scale field studies to our knowledge Mab are not yet used for detecting snails infected with schistosomes.

The use of DNA probes for detecting parasites was gaining momentum at the time our study was started. As far as schistosomes are concerned, cloned ribosomal gene fragments were employed for determining species, strain and sex of schistosomes (11,12). Specific identification was possible by different banding patterns exhibited when schistosomal DNA was digested by restriction enzymes then run electrophoretically and underwent southern blot analysis with rDNA probes. We did not consider this approach suitable for testing multiple samples. Furthermore, since ribosomal genes are represented by only 100 copies in the S. mansoni genome this test was not expected to provide a sufficiently high sensitivity. We were therefore seeking a suitable probe that is highly represented in the schistosomal genome, exhibits species specificity and can be used in a dot hybridization test. Such highly repeated sequences were identified in filariae (13). It was also important to develop a simple procedure for preparing snails for dot hybridization because extraction of DNA from each snail by the conventional method (14) involves multiple steps (including mechanical disruption, Proteinase K digestion, Phenol extraction and alcohol precipitation) and is not practical for examining multiple samples. The use of rDNA probes for detecting infected snails by dot hybridization was attempted but detection sensitivity was low (14) and species specificity cannot be accomplished by rDNA probe in this test configuration due to the presence of highly conserved sequences. Species specific schistosomal sequences that are unique to females (15) cannot be considered for practical purposes not only because

this sequence is represented by only 75 copies/genome but mainly because most snail infections in nature araise from a single micracidium and therefore constitutes a single sex. The large proportion of snails infected with male schistosomes is bound to be missed if these probes were to be used. Since our probe represents a highly repeated DNA sequence that is present in both sexes and is species specific, it appears to be suitable for detecting infected snails.

The project was initially supported for 3 years by AID/PSTC and was extended for about 2 1/2 more years. This was made possible partly by a no-cost extention of the initial AID/PSTC grant, and partly by using funds from the NIAID Egypt/Israel/USA project on vector borne diseases in the Middle East. Part of these funds, intended for developing a DNA probe for detecting W. bancrofti infection, were used for doing a similar study on S. mansoni as a model system pending supply of W. bancrofti material by the Egyptian team. Completion of the study on S. mansoni DNA probes enabled us later on to proceed and identify highly repeated sequences in W. bancrofti (work now in progress).

Methods and Results. This project, produced 4 major publications entitled as follows (and arranged according to a subject sequence).

a) Schistosoma mansoni antigens recoognized in Biomphalaria glabrata hemolymph by monoclonnal antibodies. A. J. Trop. Med. Hyg. 40:605-612 1989.

This publication describes the production of monoclonal antibodies and the identification of the ones that are specific for S. mansoni antigens within infected snails. These Mab were employed in a simple ELISA configuration for identification of abundant schistosomal antigens in extracts and hemolymph of infected B. glabrata during prepatency and patency. Characterization of the antigens recognized by the selected Mab was carried out by Western blotting, and their glycoprotein nature shown by their periodate sensitivity.

b) Identification of snails infected with schistosomes by ELISA employing monoclonal antibodies: Schistosoma mansoni in laboratory snails (Biomphalaria glabrata) and in field snails (Biomphalaria pfeifferi) from Kenya.

This publication described the employment of Mab (see above) in a simple one-step ELISA for indentification of infected snails by detection of schistosomal antigens in the haemolymph of infected snails. Infected laboratory snails were differentially identified from uninfected laboratory snails (B. glarbata) during preparency and through patency with 100% sensitivity and specificity. Infected field snails (B. pfeifferi) were differentially

identified with 100% sensitivity and specificity from uninfected field snails and from snails naturally infected with other trematodes (eschinostomes and strigeids). Prepatent infections were readily identified in field snails.

c) Detection of Schistosoma mansoni DNA in extracts of whole individual snails by dot hybridization. Parasitrol. Res. 74:97-100 (1987).

A simple procedure was developed for preparing snails for dot hybridization. The procedure involves tituration of each snail in presence of NaOH+detergent following by neuralization, heating and centrifugation and finally spotting.The procedure enables easy preparation of multiple samples and can be considered suitable for field work. Using a total S. mansoni DNA probe it was possible to detect infected snails as soon as one week after their exposure to miracidia. No cross reaction was observed with normal snail extracts.

d) Highly repeated short DNA sequences in the genome of Schistosoma mansoni recognized by a species specific probes. Mol. Biochem. Parasitol., in press.

A cloned 0.6 kb DNA sequence of S. mansoni contains 121 bp tandem repeats and comprises at least 12% of the schistosomal genome of both sexes. It exhibits a high degree of species specificity and could detect with high sensitivity schistosomal DNA sequences in infected snails.

Impact, Relevance and Technology Transfer: The findings of our research clearly demonstrate the feasibility of the use of monoclonal antibodies and DNA probes for the identification of infected snails. Cercarial shedding, which is currently used for this purpose, is expected to prevail as the major test for routine identification of snails infected with S. mansoni. However, our approach will be considered for routine use if the detection procedure is further simplified and the test made cost effective. In practice even if "molecular identification" of infected snails is made cost effective and usable by community health workers in endemic areas it is not expected to replace shedding tests for identifying snails patently infected with S. mansoni. It may, however, be useful in these areas for periodic quality control of shedding tests in particular where the routine differentiation of S. mansoni cercariae from a large variety of non-human cercariae is difficult. Adaptation of "molecular" approach for monitoring S. haematobium infection in snails will make it the approach of choice because shedding tests in this case do not provide specific identification of the human schistosome vis a vis a number of animal schistosomes which can develop in the same snail species. The immediate use of our Mab for the identification of schistosomal antigens (S. mansoni) in snail hemolymph can be considered for research purposes wherever accurate data are required on the effective influx of the parasite from the vertebrate to the invertebrate hosts of S. mansoni. This should be possible as soon as

our results gain credence by large scale trials in which the prepatent/patent infection ratio should be examined as a measure for such an influx.

The use of Mab for detecting infected snails may gain a quicker adaptation for field studies in LDC sinced ELISA is a well rehearsed routine procedure in many countries where schistosomiasis is endemic. DNA hybridization technology on the other hand, will be adopted only if non-radioactive probes prove to be effective. Such non-radioactive detection should constitute part of the further research and development required.

The introduction of modern diagnostic biotechnology for routine diagnostic/epidemiological purposes of parasitic diseases (schistosomiasis being only one example) will require some organizational changes. Public Health Laboratories will have to acquire these technologies by close collaboration with research institutions who may already be involved in the use of the relevant technologies. In this context training as well as redefinition of job responsibilities of technical and scientific staff will become an important matter. Technical staff should be given avenues for further training through acquisition of an academic degree since much of the modern diagnostic biotechnology requires a broader academic training. Thus, more academicians will be required at the laboratory bench instead of the managerial desk. Training on location should receive more emphasis. Accordingly, research institutions and central Public Health Laboratories should consider organizing central facilities as well as suitable personnel

(including invitated scientists) for providing a much needed bridge for technology acquisition. Such facilities will also serve as convenient central training sites.

Specifically relating to technology transfer within the context of this project it should be taken into account that the establishment of a hybridoma unit in a developing country is dependent on the availability of suitable space and supplies. A separate room with UV sterilization, and minimum access to unauthorized personnel is required to avoid contamination which is much more prevalent in the tropics. Reliable supply of media, immunochemicals, plasticware, etc., is a must for a hybridoma unit to be operational on a continuous basis. This is likely to require special logistic arrangements in Kenya as well as in many other LDC.

Project Activities Ouputs: Results of this project were presented in the following meetings:

- Annual meetings of the Israel Society of Parasitology (1986, 1988, 1989).
- The 3rd Mediterranean Conference for Parasitology (Jerusalem 1987)
- Meetings of participants of the Egypt/Israel/USD NIAID Regional Project of Vector-Borne Diseases in the Middle East (Tel Aviv 1988, Taba 1990).
- AID Conference on Biotechnology in Agriculture and Medicine (National Academy of Science, Washington, D.C. U.S.A., 1988).
- Seminar at the Biomedical Research Centre, Kenya Medical Research Institute (Nairobi, 1989).

Training was provided to one Kenyan Senior technologist on theory and practice of the hybridoma technology, and on antigenic analysis using monoclonal antibodies. It took place in Jerusalem for a 4 months period. A hybridoma unit was established at KEMRI (in the Biomedical Research Centre) and initial cultures and fusion experiments carried out. A contamination-free room is yet to be arranged for this unit and logistic problems of supplies are yet to be solved before it can be operational on a continuous basis.

This project resulted in 4 major publications and 5 abstracts as follows:

Major publications:

Hamburger, J., Weil, M. and Pollack, Y. 1987. Detection of Schistosoma mansoni DNA in extracts of whole individual snails by dot hybridization. Parasitol. Res., 74:97-100.

Hamburger, J., Weil, M., Anton, M. and Turetsky, T. 1989. Schistosoma mansoni antigens recognized in Biomphalaria glabrata hemolymph by monoclonal antibodies. Am. J. Trop. Med. Hyg., 40:605-612.

Hamburger, J., Weil, M., Turetzky, T., Ouma, J.H., Koech, D.K., Klumpp, R., Siongok, T.K.A. and Sturrock, R. 1989. Identification of snails (Biomphalaria) infected with Schistosomes by ELISA employing monoclonal antibodies: Schistosoma mansoni in laboratory snails (B. glabrata) and in field snails (B. pfeifferi) from Kenya. Am. J. Trop. Med. Hyg., 40:613-619.

Hamburger, J., Turetzki, T., Kapeller, I. and Deresiewicz, R. 1991. Highly repeated short DNA sequences in the genome of Schistosoma mansoni recognized by a species specific probe. Mol. Biochem. Parasitol., in press.

Abstracts

Pollack, Y. and Hamburger, J. 1986. Detection of Schistosoma mansoni infection in snails by the use of DNA probes. Isr. J. Med. Sci. 22(11):851.

Hamburger, J. and Weil, M. 1987. Immunodetection of Schistosoma mansoni infection in snails. Proceedings of the 3rd Mediterranean Conference of Parasitology, p. 77.

Hamburger, J., Weil, M., Turetsky, B., Anton, M., Ouma, J.H., Siongok, T.K.A., Koech, D.K. and Sturrock, R. 1988. The potential epidemiological usefulness of an ELISA employing monoclonal antibodies for the identification of schistosome-infected snails. Isr. J. Med. Sci.

Hamburger, J., Weil, M., Turetsky, B., Deresiewicz, R., Anton, M., Ouma, J.H., Siongok, T .K.A., Koech, D.K. and Sturrock, R. 1988. Detection of snails infected with Schistosoma by the use of antibodies and DNA probes. PSTC conference on Biotechnology for Health and Agriculture. Washington, D.C.

Hamburger, J., Turetzky, T., Deresiewicz, R. and Pollack, Y. 1990. Potential diagnostic significance of a schistosomal DNA probe representing highly repeated species-specific sequences. Isr. J. Med. Sci., In press.

Copies of the major publications are herewith enclosed.

Project Productivity: The project accomplished all of the proposed goals.

Future Work: The project is expected to lead to future work on several lines.

1) Development of Mab and DNA probes for detecting snails infected with other human schistosomes.

2) Development of non-radioactive, cost effective procedures for detecting prepatent infection in snails infected with S. mansoni.

3) Elucidation of strain specificity of the DNA probe representing highly repeated sequences of S. mansoni This study is expected to include elucidation of consensus sequences from various strains in search of markers for strain associated differences in pathogenicity, response to drugs and various aspects of host-parasite compatibility.

4) The use of the Mab developed in this project in search of putative receptors on snail hemocytes responsible for recognition of intramolluscan S. mansoni larvae and subsequent cytoadherence. This is part of an overall study for elucidating molecular aspects of S. mansoni-snail interactions.

Literature Cited

1. Capron, A., Biguet, J., Rose, F., Vernes, A. 1965. Les antigens de Schistosoma mansoni. II. Etude immunoelectrophoretique comparee de divers stades larvaires et des adults des deux sexes. Aspects

immunologiques des relations hote-parasite de la cercarie et de l'adulte de S. mansoni. Ann. Inst. Pasteur Immunol. (Paris) 109:798-810.

2. Kemp, W.M., Greene, N.D., Damian, R.T. 1974. Sharing of Cercarienhullen Reaktion antigens between Schistosoma mansoni cercariae and adults and uninfected Biomphalaria pfeifferi. Am. J. Trop. Med. Hyg. 23:197-202.

3. Jackson, T.F. 1976. Intermediate host antigens associated with the cercariae of Schistosoma haematobium. J. Helminthol. 50:45-47.

4. Yoshino, T.P., Cheng, T.C. 1978. Snail host-like antigens associated with the surface membranes of Schistosoma mansoni miracidia. J. Parasitol. 64:752-754.

5. Lie, K.J. 1982. Swellengrebel lecture: survival of Schistosoma mansoni and other trematode larvae in the snail Biomphalaria glabrata. A discussion of the interference theory. Trop. Geogr. Med. 34:111-122.

6. Stek M. Jr., Coulis, P.A., Boctor, F.N., Pelley, R.P. 1983. Reactivity of anti-MSA-1 monoclonal antibody with schistosomal and non-schistosomal antigenic extracts. Lancet 2:522-523.

7. Bayne, C.J., Loker, E.S. Yui, M.A. 1986. Interactions between the plasma proteins of Biomphalaria glabrata (Gastropoda) and the sporocyst tegument of Schistosoma mansoni (Trematoda). Parasitology 92:653-664.

8. Dissous, C., Grzych, J.M., Capron, A. 1986. Schistosoma mansoni shares a protective oligosaccharide epitope with freshwater and marine snails. Nature 323:443-445.

9. Bayne, C.J., Boswell, C.A., Yui, M.A. 1987. Widespread antigenic cross-reactivity between plasma proteins of a gastropod, and its trematode parasite. Dev. Comp. Immunol. 11:321-329.

10. Abdul-Salam, J.M., Michelson, E.H. 1983. Schistosoma mansoni: immunofluorescent detection of its antigen reacting with Biomphalaria glarbata amoebocytes. Exp. Parasitol. 55:132-137.

11. McCutchan, T.F., Simpson, J.G., Mullins, J.A., Sher, A., Nash, T.E., Lewis, F. and Richards, C. 1984. Differentiation of schistosomes by species, strain and sex by using cloned DNA markers. Proc. Natl. Acad. Sci. USA, 81:889-893.

12. Rollinson, D., Walker, T.K. and Simpson, A.J.G. 1986. The application of recombinant DNA technology to problems of helminth identification. Parasitology 91:853-954.

13. Piessens, W.F., McReynolds, L.A. and Williams, S.A. 1987. Highly repeated DNA sequences as species specific probes for Brugia. Parasitol. Today 3:387-389.

14. Rollinson, D., Walker, T.K., Simplson, A.J.G. 1986. The application of recombinant DNA technology to problems of helminth identification. J. Parasitol. 912:553-571.

15. Walker, T.K., Rollinson, D. and Simpson, J.G . 1989. A DNA probe from Schistosoma mansoni allows rapid determination of the sex of larval parasites. Mol. Biochem. Parasitol. 33:33-100.

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SCHISTOSOMA MANSONI ANTIGENS RECOGNIZED IN *BIOMPHALARIA GLABRATA* HEMOLYMPH BY MONOCLONAL ANTIBODIES

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Abstract. In order to identify and characterize *Schistosoma mansoni* antigens in *Biomphalaria glabrata*, we examined 19 murine monoclonal antibodies (Mabs) for specific binding to schistosome larvae. None of the murine Mabs induced by infection or by immunization with a crude cercarial antigen (CCA) served this purpose. Two Mabs out of 9 (KCSme22-3 and KCSme22-4) induced by soluble egg antigens reacted with CCA but not with normal snail (NSN) extract. We selected these 2 for studies on detection and characterization of schistosomal antigens in snails. When employed in an ELISA, they differentially detected schistosomal antigens in extracts and cell-free hemolymph (plasma) of infected snails. The selected Mabs bind to cercarial surface as demonstrated by the indirect fluorescent antibody technique (IFAT) with paraformaldehyde-fixed cercariae. The epitopes corresponding to the selected Mabs are periodate sensitive, suggesting the glycoprotein nature of the antigens recognized. Immunoblotting analysis employing the selected Mab revealed 1 antigen in CCA (M. = 205 kDa) and 3 antigens in snail plasma (M. = 220 kDa, 130 kDa, and 135 kDa). Schistosomal antigens were first detectable in the snails' plasma 2 weeks after snail infection, and their quantity increased afterwards.

The widespread antigenic cross-reactivity which exists between *Schistosoma mansoni* and its host *Biomphalaria glabrata*[1] is possibly one way the parasites evade snail defenses.[2] Parasite specific antigens, on the other hand, have been suggested as possible targets of specific recognition of the parasite as foreign by snail hemocytes,[3] recognition being an important initial step in mobilization of snail defenses against schistosome infection.

Little is known of the identity and characteristics of schistosome specific antigens within their intermediate snail hosts, despite the importance of such knowledge for the elucidation of schistosome-snail interactions. Schistosome specific antigens have been detected in hemolymph and hemocytes of infected *B. glabrata* by employing suitably absorbed infected mouse serum as antigen detector,[3] but none have yet been defined. Tegmental surface proteins of *S. mansoni* sporocysts have been analyzed by monoclonal antibodies (Mabs), taking into account their possible role as targets of snail defenses.[4] However, their specificity in relation to snail antigens has not been defined. The employment of Mabs for identification and characterization of schistosome specific antigens in infected *B. glabrata* is the subject of this paper.

MATERIALS AND METHODS

Parasite and hosts

Schistosoma mansoni (Egyptian strain) was maintained in outbred albino female mice and in snails (*Biomphalaria glabrata*) as previously described.[4] Snails were infected individually by exposure to 10 miracidia each.

Antigenic preparations

S. mansoni ova were harvested from intestines of infected mice by homogenization, trypsinization, and differential sieving. Soluble egg antigen (SEA) was prepared from the ova by homogenization and ultracentrifugation.[5,6]

A crude cercarial antigen (CCA) was prepared by sonication of lyophilized cercariae in phosphate buffered saline (PBS), pH 7.2. Sonication was carried out with a Sonicator W-225 (Ultrasonic Inc.) in 3 cycles of pulses (40% cycle) for 3 min each.

Extracts from normal or infected snails were prepared in a test tube by mechanical disruption of whole snails with motor driven wooden applicators at 1,800 rpm for 2 min. Disruption of snails was followed by centrifugation at 12,000

20'

rpm (Mikrotiter Hettich, West Germany) for 15 min.

Snail hemolymph was drawn out with a mouth operated suction tube to which a 25 G needle was attached. The snails were pierced near the innermost coil of the shell. Snail plasma was obtained by centrifuging the hemolymph for 5 min at 12,000 rpm (Mikrotiter Hettich). Protein concentration of the various antigenic preparations was determined by the method of Lowry and others.[19]

Monoclonal antibodies

Three series of Mabs, differing in the nature of antigenic stimulation given to the splenocytes donors (BALB/c female mice, 6 weeks old), were prepared. For the first series, mice were infected by subcutaneous injection of 50 *S. mansoni* cercariae; spleen cells were taken for fusion 6 weeks later. For the second series, mice were immunized to CCA: an ip injection of 100 μg CCA incorporated in Freund's complete adjuvant was followed 2 weeks later by 50 μg CCA incorporated in Freund's incomplete adjuvant. The mice were then allowed to rest for at least 1 month; 4 days before splenocytes were taken for fusion, the mice received 50 μg CCA without adjuvant. For the third series, mice were immunized against egg antigens by a weekly ip injection of 5,000 live *S. mansoni* ova for 3 consecutive weeks. After a month's rest, the mice received an iv injection of 20 μg SEA 4 days before splenocytes were taken for fusion.

Mabs were prepared according to established protocols.[17,18] Myeloma cells, line x63-Ag8-653,[17] were fused with splenocytes at a ratio of 1:5 with polyethyleneglycol (M. 3,600). Subcloning was carried out by limiting dilution, and ascitic fluids were prepared by ip injection of hybridoma cells into pristane pretreated mice.

Enzyme linked immunosorbent assay

An ELISA was carried out for several purposes: the routine screening of antibody activity to homologous antigens in corresponding hybridoma supernatants and ascitic fluids, for secondary screening of Mabs in order to select those potentially suitable for specific detection of schistosomal antigens in infected snails, for the titration of selected Mabs, for the determination of the Ig isotype of selected Mabs, and for the identification of schistosomal antigens in extracts and hemolymph (or plasma) of infected snails.

Established ELISA procedures[20] were employed, with slight modifications. Briefly, wells of flexible microtiter plates (Falcon, Becton-Dickinson Labware, Oxnard, CA) were coated overnight with 65 μl/well antigen diluted in PBS to 10 μg/ml, unless otherwise indicated; 5% bovine serum albumin (BSA) was employed for the blocking of nonspecific sites by incubation for 1 hr at 37°C. Aliquots (50 μl/well) of tested culture supernatants or ascitic fluids, diluted when necessary with 5% fetal calf serum, were incubated in the antigen coated wells for 2 hr at 37°C and then for ½ hr at 4°C. The wells were washed with PBS plus 0.1% Tween 20 (PBS-T), and probing for antibody binding was routinely carried out with goat anti-mouse Ig (polyvalent) conjugated to alkaline phosphatase (Sigma Chemicals, St. Louis, MO). For determining Ig isotypes goat anti-mouse IgM (Sigma) and rabbit anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 (Litton-Biometrics) were employed. Following incubation at the described conditions, the wells were washed with PBS-T and the substrate P-nitrophenyl disodium (Sigma) was added. The color reaction was read by an ELISA reader (Titroteck Multiscan, Flow Laboratories, Scotland).

Immunochemical characterization

To determine whether epitopes corresponding to the selected Mab were carbohydrate or polypeptide in nature, we absorbed infected snail (ISN) plasma diluted 1:10 (≈50 μg/well), CCA (65 ng/well), and SEA (65 ng/well) onto a microtiter plate. Treatment with various concentrations of periodate followed, and then an ELISA was performed as previously described.[21] BSA/anti-BSA (rabbit) served as a control for periodate insensitive epitopes. A Mab (KCSmeg1) induced by the major egg glycoprotein of *S. mansoni*[22] was prepared in our laboratory and found to be directed against a carbohydrate epitope (unpublished results). KCSmeg1 was employed as a control for detecting periodate sensitive epitopes.

Indirect fluorescent antibody test

Binding of Mab to cercarial surface was investigated by an indirect fluorescent antibody test (IFAT). Cercariae were collected from infected

snails and washed 3 times with 100 ml PBS in a filtration apparatus (Sartorius, West Germany) holding a 47 mm diameter filter with a pore size of 5 μm. Washed cercariae were fixed for 15 min with 2.5% (final concentration) of neutralized paraformaldehyde in PBS. They were subsequently washed 5 times with PBS and incubated for 20 min with 0.5% BSA in PBS in order to bind any unreacted aldehyde groups. Mab diluted 1:2,000 in 0.5% BSA-PBS was incubated, for 20 min at 37°C, with 100 cercaria in a total volume of 200 μl. This was followed by 3 washes in PBS and incubation as described above with 200 μl of fluorescein-conjugated rabbit anti-mouse IgG (H+L; Bioyeda, Rehovot, Israel) diluted 1:50 with 0.02% Evans blue in PBS. Following 3 washes with PBS, the cercariae were suspended in PBS glycerol (v/v) and examined under a fluorescent microscope. Photomicrographs were taken under 400× magnification. Controls with no Mab and with irrelevant Mab were prepared and examined in parallel.

Polyacrylamide gel electrophoresis

The antigens SEA and CCA and ISN and normal snail (NSN) plasma were tested by polyacrylamide gel electrophoresis (SDS-PAGE). The various antigens were dialyzed against phosphate buffer (0.01 M, pH 7.4), and electrophoresis was carried out according to Laemmli[23] in 10% acrylamide slab gels (16 × 17 cm). About 40 μg of each antigen lane were run at 40 V overnight. Molecular weight markers (MW-SDS 200 kit and MW-SDS 70 kit, Sigma) were run in parallel.

Western blotting and immunodetection

Proteins revolved by SDS-PAGE were transferred electrophoretically onto nitrocellulose paper (NCP) by established procedures.[24,25] After the transfer, a section of the NCP containing a set of resolved antigens and molecular weight markers was stained with 0.1% amido black in a mixture of H_2O : acetic acid : methanol (4:1:5). A parallel set of resolved antigens underwent immunodetection as follows: nonspecific sites were blocked for 2 hr at 37°C by a blocking solution (5% NP-40, 0.1% Triton X-100, and 5% milk powder in PBS). The same solution was used for washing the NCP and for diluting the reagents. Mabs diluted 1:2,000 were reacted with the NCP at room temperature for 2 hr while shaking. The NCP was then washed 3 times and incubated under similar conditions with affinity-purified and alkaline phosphatase-conjugated goat antibody to mouse Fab (Bioyeda, Rehovot, Israel) diluted 1:1,000. After further washings, a color reaction was induced with a substrate mixture consisting of 40 mg naphtyl phosphate disodium (Sigma) and 100 mg Fast Red TR (Sigma) in 100 ml of 50 mM Tris, pH 9.09. The color reaction was stopped by transferring the NCP into a mixture of H_2O : acetic acid : methanol (5:1:5) and the NCP was then washed with water and dried.

RESULTS

Screening of monoclonal antibodies

A total of 19 Mabs were obtained. They were further screened by ELISA in order to select Mabs reactive with CCA but non-reactive with NSN extract. Such Mabs were considered potentially suitable for detecting schistosomal antigens in ISN extract. Of the 6 Mabs induced by infection, 4 reacted with both CCA and NSN extract and 2 reacted with neither of these antigens. Thus, none of these Mabs were suitable for our purposes. The 4 Mabs induced by CCA reacted with both CCA and NSN extract and were also unsuitable for our purposes. Of 9 Mabs induced by SEA, 3 reacted with both CCA and NSN, 4 reacted with neither of these antigens, and 2 (KCSme22-3 and KCSme22-4) reacted with CCA but not with NSN extract. These last 2 were selected for detecting schistosomal antigens in infected snails. Both these Mabs were IgM.

Quantitative aspects of Mab-antigen interactions

Binding of different dilutions of Mabs to wells exposed to different concentrations of antigens was examined by ELISA. Preliminary titration of both selected Mabs (in ascitic fluids) was carried out by ELISA with 750 ng/well CCA. A titer of 56:200 was exhibited by both. Subsequently, antibody activity of both Mabs at dilutions of 1:1,000, 1:5,000, 1:10,000, and 1:25,000 was determined with CCA at concentrations of 7.5–750 ng/well and with snail extracts (not centrifuged) at concentrations of 75–7,500 ng/well. Similar results were obtained with all Mab dilutions employed, and representative results with a 1:5,000 dilution of KCSme22-3 are presented in Figure

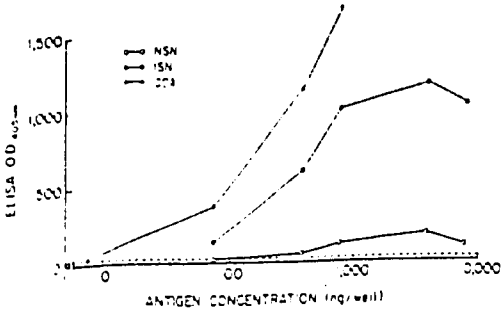

FIGURE 1. Reactivity by ELISA of Mab KCSme22-3 (at 1:5,000 dilution) with various amounts of a crude cercarial antigen (CCA), a pooled extract from normal snails (NSN), and a pooled extract of infected snails (ISN). Broken line indicates average OD readings obtained with an irrelevant Mab.

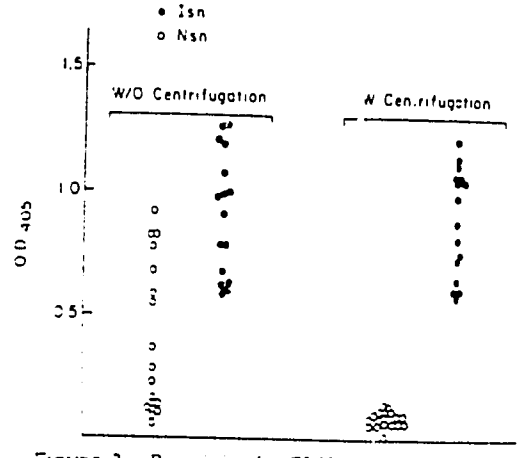

FIGURE 2. Reactivity by ELISA of extracts from individual snails with Mab KCSme22-3 and the effect of the removal of particulate material by centrifugation. Extracts of infected snails (ISN) and normal snails (NSN) were tested with (W) and without (W/O) initial centrifugation.

1. KCSme22-4 showed similar binding activity (results not shown). Mab binding to CCA and to a pool of ISN extract rose with the rise in antigen concentration (3,750–7,500 ng/well). These concentrations are about 10 times higher than those yielding maximum binding with a variety of other antigens.[9] Given this finding and taking into account that schistosomal antigens constitute only a fraction of the overall constituents in infected snail extract, these results seem to suggest that the relevant schistosomal antigens were preferentially bound to the plate. Calculations of CCA equivalents (OD with ISN/OD with CCA) at ISN extract concentrations of 75–750 ng/well indicated that, on the basis of protein concentration, only about 3 times more ISN extract was required to give antibody binding equivalent to that obtained with CCA. These results suggest the abundance of the corresponding epitopes in infected snails and/or a differentially high binding of these epitopes to the solid phase, as mentioned above.

Binding of the selected Mab to a pooled uncentrifuged extract of NSN was usually low. However, when high concentrations of pooled NSN extracts were employed, ELISA readings could occasionally reach a value several times higher than baseline values obtained with an irrelevant Mab (Fig. 1). Furthermore, when extracts of individual NSN were tested, several of them exhibited high binding (Fig. 2). This was attributed to the nonspecific binding of the Mab to particulate material present in the extract. Indeed, removal of particles by centrifugation abolished this binding (Fig. 2). Also, since centrifugation did not significantly change the binding of the Mab to the ISN extract (Fig. 2), it was assumed that the bulk of the corresponding antigens were soluble. This was supported by the finding that plasma (cell-free hemolymph) from infected snails is readily reactive with both selected Mabs.

Plasma from 8 ISN (shedding cercariae) and 8 NSN was applied to the microtiter plate at double dilutions of 1:10–1:5,120 (corresponding to protein concentrations of 51.82 = 16.6–0.009 = 0.001 μg/well for ISN plasma and 62.39 = 16.6–0.012 = 0.003 μg/well for NSN plasma). The results of this test employing KCSme22-3 are presented in Figure 3A. Similar results were obtained with KCSme22-4 (results not shown). Mab binding to NSN plasma was invariably very low. High reactivity was exhibited with plasma from 4 of 8 ISN at all dilutions, and a prozone effect was observed. Plasma from the other 4 ISN in this group exhibited lower reactivity. Plasma dilutions of 1:160, corresponding to an average protein content of ≈3,250 ng/well, gave the maximal average binding of Mab. Since binding maxima were obtained with pooled ISN extracts of a closely similar concentration (3,750 ng/well) (Fig. 1), these results again suggest that a substantial proportion of the schistosomal epitopes detected in ISN by the selected Mab are present in the plasma.

The appearance of schistosomal antigens in snails' plasma collected 1–6 weeks after exposure to miracidia was detected ELISA. Detectable Mab

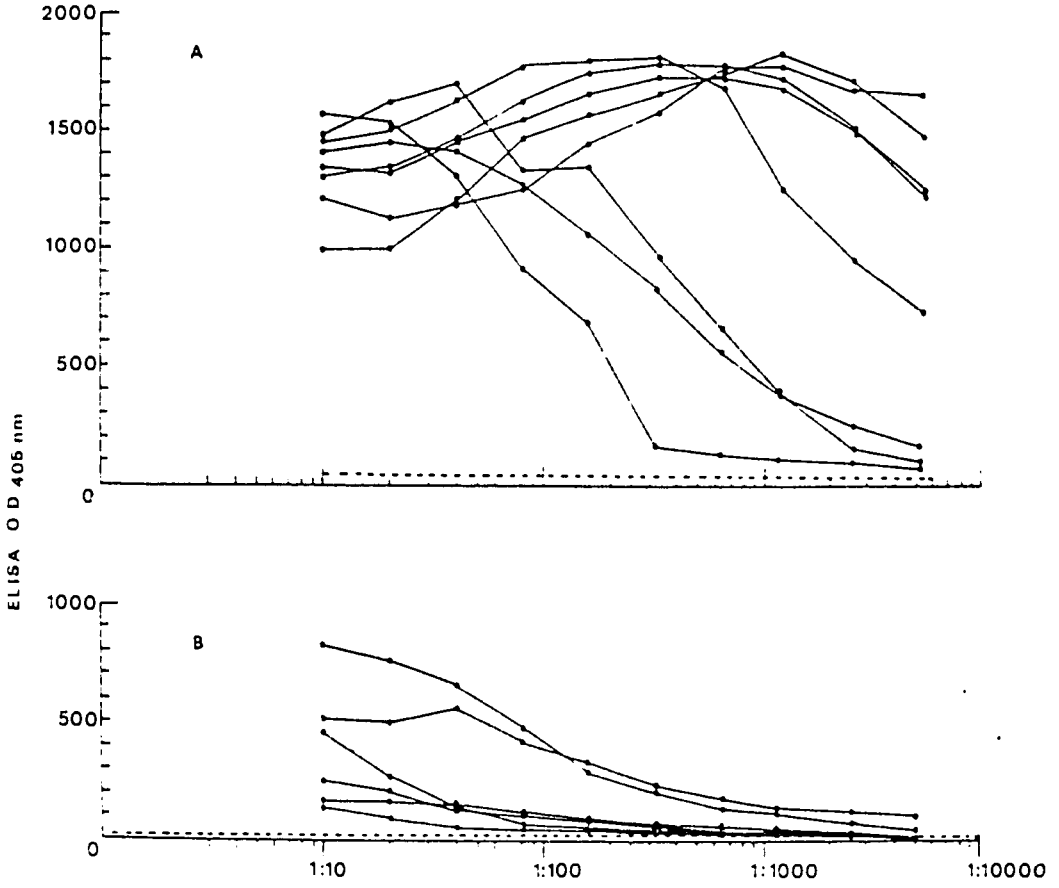

FIGURE 3. Reactivity by ELISA of cell-free hemolymph (plasma) from 3 snails harboring patent infection (A) and 6 snails harboring prepatent infection (B) of 2 weeks duration. Horizontal axis indicates dilution of snail plasma. Plasma from individual snails at doubling dilutions (1:10–1:5,120) was applied onto microtiter plate wells, and Mab KCSme22-3 was employed as an antigen detecting reagent. Broken line indicates the upper limit of ELISA OD readings with plasma from the same number of normal snails (8 and 6, respectively).

binding was exhibited starting at 2 weeks (results not shown). In our infected snails, this time coincides with the release and migration of daughter sporocysts. Binding curves obtained with plasma from 6 snails infected for 2 weeks are presented in Figure 3B. Prepatent infection plasma exhibited much lower Mab binding than did patent infection plasma (Fig. 3A). These results indicate that the quantity of the corresponding epitopes increases as infection progresses from prepatency to patency.

Characterization of schistosomal antigens detected by the selected Mabs

Periodate sensitivity of epitopes corresponding to KCSme22-3 and KCSme22-4 is presented in Table 1. Treatment of SEA, CCA, and ISN plasma with a suitable concentration of periodate (<0.1 mM for SEA, <5 mM for CCA, and 5–10 mM for ISN) abolished the binding of both selected Mabs to these antigens. The observed differences in periodate sensitivity may represent differences in consumption of periodate depending on the total concentration of the absorbed antigen and on differences in the density of periodate sensitive sites. Based on these results, we assume that antigens exposing epitopes corresponding to both selected Mabs are glycoproteins.

Western blot analysis of infected snail plasma constituents resolved by SDS-PAGE was carried out with both selected Mabs. Results obtained with KCSme22-3 are presented in Figure 4. Mul-

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TABLE I

Periodate sensitivity of epitopes corresponding to Mab KCSme22-3 and KCSme22-4

Antibody	Antigen	ELISA OD with periodate treated antigens at various periodate concentrations (mM)				
		0	0.1	5	10	20
KCSme22-3	SEA	0.505	0.003	0	0	0
	CCA	0.675	0.726	0.045	0.027	0.043
	ISP	1.721	1.722	0.179	0	0.022
KCSme22-4	SEA	0.562	0	0	0	0
	CCA	0.688	0.729	0.045	0.027	0.043
	ISP	1.590	1.619	0.431	0.031	0.035
KCSmeg1 (positive control)	SEA	0.721	0	0	0	0
Rabbit anti-BSA (negative control)	BSA	0.626	0.663	0.646	0.758	0.536

tiple plasma constituents with M. of >200 kDa–35 kDa were resolved by SDS-PAGE (Fig. 4, lanes g, h). CCA was resolved into multiple constituents within >200 kDa–24 kDa (Fig. 3, lane f). Immunodetection with KCSme22-3 revealed 1 constituent at 205 kDa in CCA (Fig. 4, lane b) and 3 schistosomal constituents of 220 kDa, 180 kDa, and 135 kDa in the plasma of ISN (Fig. 3, lane d). Plasma from NSN did not exhibit any binding of Mab as expected (Fig. 4, lane c). A similar pattern was obtained with KCSme22-4 (results not shown).

Paraformaldehyde-fixed cercariae gave strong fluorescence on cercarial body and tail when tested by IFAT with both selected Mabs, suggesting that the corresponding 205 kDa cercarial antigen is associated with the entire cercarial surface. Representative results with KCSme22-3 are presented in Figure 5.

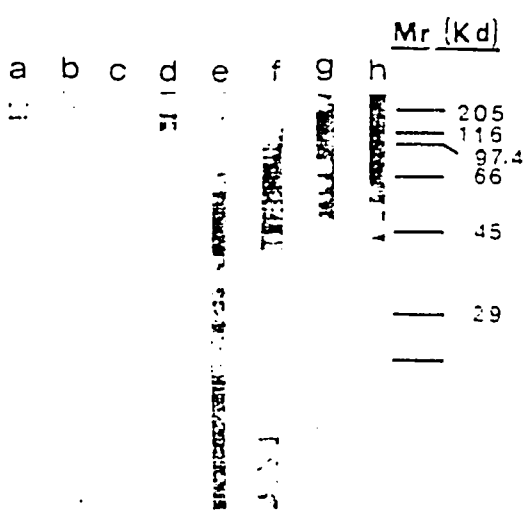

FIGURE 4. Western blots (lanes a–d) were done with Mab KCSme22-3 as an antigen detecting reagent. They correspond to SDS-PAGE of SEA, CCA, NSN plasma, and ISN plasma (lanes e–h, respectively).

DISCUSSION

In the present study, we have employed Mabs in search of parasite specific antigens in *B. glabrata* infected with *S. mansoni*. In view of the widespread cross-reactivity of schistosomal antigens with their snail host, it is not surprising that among a total of 13 Mabs which reacted with cercarial antigens (CCA), 11 also reacted with extracts from normal snails (NSN). Antigen sharing between snails and schistosome larvae is thought to be largely due to acquisition of snail constituents by the parasites and perhaps also by true antigenic mimicry. The antigen sharing found between schistosome eggs and snail antigens, as detected by 3 of 9 Mabs which were induced by SEA, cannot be explained as being due to acquisition, since schistosome eggs do not come in contact with snail tissues. Therefore, it may be assumed that the corresponding epitopes are synthesized by schistosome eggs. Such shared antigens, presumably synthesized by the parasite, are particularly interesting in the context of the molecular evolution of the host-parasite adaptation.

Cross-reactivity between egg antigens and cercarial antigens was exhibited by 5 of 9 Mabs induced by SEA. Among these, KCSme22-3 and KCSme22-4 were parasite specific and identified carbohydrate epitopes (Table I) in schistosomal antigens of high relative molecular mass (Fig. 4). Both selected Mabs were IgM and gave similar results by all tests employed (ELISA, Western blotting, IFAT). Whether they are directed against the same epitope remains to be determined by

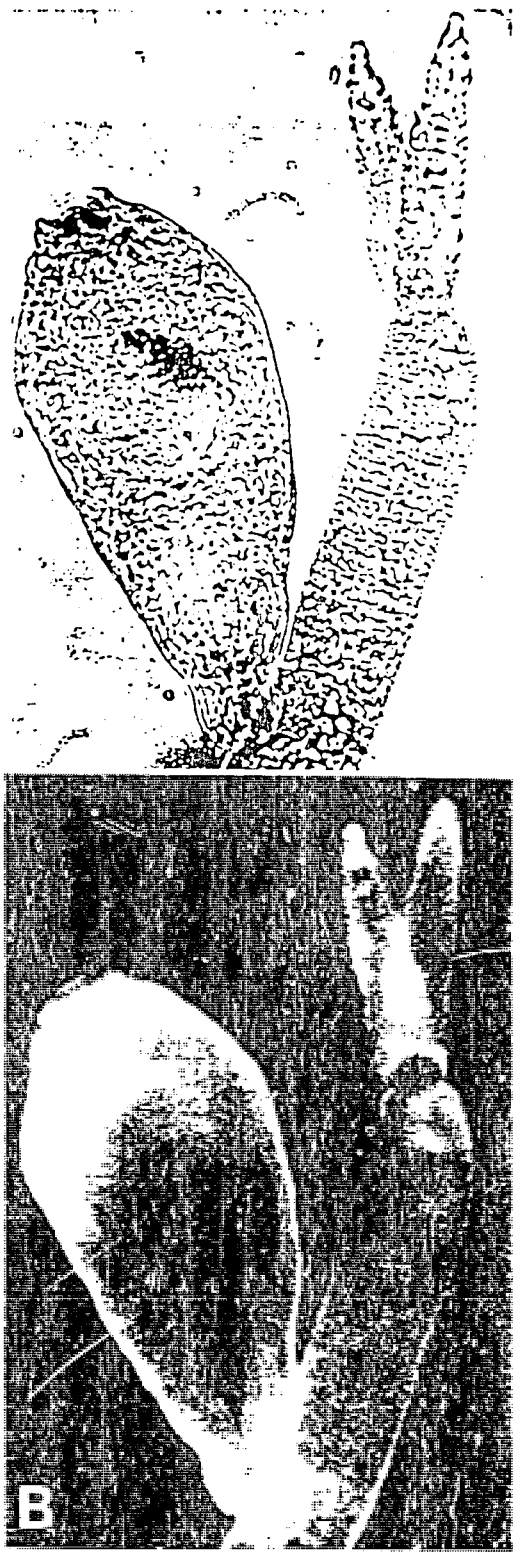

FIGURE 5. Photomicrograph of paraformaldehyde-treated *S. mansoni* cercariae processed for immunofluorescence staining using Mab K7Sme22-2 as primary antibody. A, Bright field. B, Fluorescence.

epitope-specificity analysis. Since the 3 antigens detected in the snails' plasma appear to be different in molecular mass from the 1 present in CCA (Fig. 4), it can be tentatively assumed that they are not derived from cercariae. This assumption is supported by the finding that these antigens first appear in the plasma 2 weeks after snail infection, before the development of cercariae in our infected snail colony. Since their quantity increases from prepatency to patency (Fig. 3), it can be assumed that they are released in a progressive manner as an outcome of the normal development of the parasite, perhaps during the release of larvae from mother and daughter sporocysts.

The parasite specificity of the schistosomal antigens detected in the snail suggests that they may be foreign to the snail, but whether they can be recognized as such by the snail remains to be determined. At present these parasite specific epitopes and the corresponding Mabs can be considered as candidate reagents for identifying putative receptors mediating recognition of parasite antigens by snail hemocytes.[12] Whether these epitopes play a role in the regulation of snail defenses is unclear. Since these epitopes are parasite specific, they cannot possibly contribute to immune-evasion mechanisms based on antigen sharing.[1] On the other hand, since parasite-specific epitopes appear to be abundant in infected snail's plasma (Fig. 3), they may play a role in blocking hemocyte activity by saturating putative receptors.[13]

From a practical point of view, several features of the Mabs and the immunoassay described for the detection of parasite specific antigens in infected snails suggest the feasibility of an immunodetection approach for identifying infected snails. The results of a preliminary evaluation of this approach for epidemiological purposes, where the identification of schistosome-infected snails was required, follow this article.[17]

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REFERENCES

1. Capron A, Biguet J, Rose F, Vernes A, 1965. Les antigens de Schistosoma mansoni. II. Etude immunoelectrophoretique comparee de divers

stades larvaires et des adultes des deux sexes. Aspects immunologiques des relations hote-parasite de la cercarie et de l'adulte de S. mansoni. *Ann Inst Pasteur Immunol Paris 109*: 798–810.

2. Kemp WM, Greene ND, Damian RT. 1974. Sharing of Cercarienhullen Reaktion antigens between Schistosoma mansoni cercariae and adults and uninfected Biomphalaria pfeifferi. *Am J Trop Med Hyg 23*: 197–202. UI:4126081
3. Jackson TF. 1976. Intermediate host antigens associated with the cercariae of Schistosoma haematobium. *J Helminthol 50*: 45–47. UI:761684
4. Yoshino TP, Cheng TC. 1978. Snail host-like antigens associated with the surface membranes of Schistosoma mansoni miracidia. *J Parasitol 64*: 752–754. UI:7324438
5. Lie KJ. 1982. Swellengrebel lecture: survival of Schistosoma mansoni and other trematode larvae in the snail Biomphalaria glabrata. A discussion of the interference theory. *Trop Geogr Med 34*: 111–122. UI:6301749
6. Stek M Jr, Coulis PA, Boctor FN, Pelley RP. 1983. Reactivity of anti-MSA-1 monoclonal antibody with schistosomal and non-schistosomal antigenic extracts. *Lancet 2*: 522–523. UI:8329631
7. Bayne CJ, Loker ES, Yui MA. 1986. Interactions between the plasma proteins of Biomphalaria glabrata (Gastropoda) and the sporocyst tegument of Schistosoma mansoni (Trematoda). *Parasitology 92*: 653–664. UI:3628631
8. Dissous C, Grzych JM, Capron A. 1986. Schistosoma mansoni shares a protective oligosaccharide epitope with freshwater and marine snails. *Nature 323*: 443–445. UI:3701480
9. Bayne CJ, Boswell CA, Yui MA. 1987. Widespread antigenic cross-reactivity between plasma proteins of a gastropod, and its trematode parasite. *Dev Comp Immunol 11*: 321–329. UI:3730507
10. Bayne CJ, Loker ES, Yui MA, Stephens JA. 1984. Immune-recognition of Schistosoma mansoni primary sporocysts may require specific receptors on Biomphalaria glabrata hemocytes. *Parasite Immunol 6*: 519–528. UI:6511219
11. Meuleman EA, Bayne CJ, van der Knaap WP. 1987. Immunological aspects of snail-trematode interactions. *Prog Clin Biol Res 233*: 113–127. UI:3723109
12. Abdul-Salam JM, Michelson EH. 1982. Schistosoma mansoni: immunofluorescent detection of its antigen reacting with Biomphalaria glabrata amoebocytes. *Exp Parasitol 55*: 132–137. UI:3310555
13. Boswell CA, Yoshino TP, Dunn TS. 1987. Analysis of tegumental surface proteins of Schistosoma mansoni primary sporocysts. *J Parasitol 73*: 778–786. UI:3731098
14. Hamburger J, Lustigman S, Siongok TK, Duma JH, Mahmoud AA. 1982. Characterization of a purified glycoprotein from Schistosoma mansoni eggs: specificity, stability, and the involvement of carbohydrate and peptide moieties in its serologic activity. *J Immunol 128*: 1864–1869. UI:8214372
15. Boros DL, Warren KS. 1970. Delayed hypersensitivity-type granuloma formation and dermal reaction induced and elicited by a soluble factor isolated from Schistosoma mansoni eggs. *J Exp Med 132*: 488–507. UI:7208201
16. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin Phenoreagent. *J Biol Chem 193*: 265–275.
17. Gafre G, Milstein C. 1981. Preparation of monoclonal antibodies: strategies and procedures. *Meth Enzymol 73*: 3–46.
18. Goding JW. 1983. *Monoclonal antibodies: principals and practice*. London: Academic Press, Inc.
19. Kearney JF, Radbruch A, Liesegang B, Rajewsky K. 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J Immunol 123*: 1548–1550. UI:3000756
20. Voller A, Bidwell DE, Bartlett A. 1977. *The enzyme linked immunosorbent assay (ELISA) (made with extracts of microplate application)*. Guernsey, Great Britain: Dynatech Europe, Borough House.
21. Woodward MP, Young WW Jr, Bloodgood RA. 1985. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *J Immunol Methods 78*: 143–153. UI:3515911
22. Lustigman S, Mahmoud AA, Hamburger J. 1985. Glycopeptides in soluble egg antigen of Schistosoma mansoni: isolation, characterization, and elucidation of their immunochemical and immunopathological relation to the major egg glycoprotein (MEG). *J Immunol 134*: 1961–1967. UI:3510629
23. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*: 680–685. UI:5432063
24. Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Nat Acad Sci USA 76*: 4350–4354. UI:3005673
25. Burnette WN. 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. UI:6125363
26. Yoshino TP, Bayne CJ. 1983. Mimicry of snail host antigens by miracidia and primary sporocysts of Schistosoma mansoni. *Parasite Immunol 5*: 317–328. UI:6324617
27. Hamburger J, Weil M, Turetzky T, Duma JH, Koech DK, Klumpp R, Siongok TKA, Sturrock RD. 1988. Identification of snails infected with schistosomes by ELISA employing monoclonal antibodies: Schistosoma mansoni in laboratory snails (Biomphalaria glabrata) and in field snails (Biomphalaria pfeifferi) from Kenya. *Am J Trop Med Hyg 40*: 613–619.

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IDENTIFICATION OF SNAILS INFECTED WITH SCHISTOSOMES BY ELISA EMPLOYING MONOCLONAL ANTIBODIES: *SCHISTOSOMA MANSONI* IN LABORATORY SNAILS (*BIOMPHALARIA GLABRATA*) AND IN FIELD SNAILS (*BIOMPHALARIA PFEIFFERI*) FROM KENYA

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Abstract. An enzyme-linked immunosorbent assay (ELISA) employing monoclonal antibodies was used for detecting *Schistosoma mansoni* antigens in hemolymph of laboratory snails (*Biomphalaria glabrata*) ~~in Keny~~a. Infected laboratory snails shedding cercariae were differentially identified by ELISA from uninfected snails with 100% sensitivity and specificity. Prepatent infections were detected by ELISA from 2 weeks after exposure to miracidia. Thus, ELISA revealed infection 3 weeks before maximal patency was reached (5–6 weeks post-exposure).

Infected field snails (*B. pfeifferi*) shedding cercariae were differentially identified by ELISA, with 100% sensitivity and specificity, from uninfected field snails and from snails naturally infected with other trematodes (echinostomes and strigeids). Prepatent infections with *S. mansoni* were readily identified by ELISA in field snails. A case is demonstrated where infection rate, as determined by shedding test alone, was 9.8%, whereas the combined figure of prepatent and patent infection rates was 22.9%.

The transmission of schistosomiasis is affected by a variety of factors relating to human infection and behavior, environmental conditions, and snail bionomics and infection.[1] Since information on transmission of schistosomiasis is important for designing control projects and for evaluating the effect of their application, upscaling of transmission monitoring is obviated by the increased use of antischistosomal drugs in community control campaigns.[2] Furthermore, should new advances in antischistosomal vaccine development lead to trials of a vaccine in human populations, more transmission monitoring is likely to follow. Identification of active transmission sites by community surveys and studies on human behavior is expensive and often results in a decline in community participation.[3,4] On the other hand, snail sites can be sampled more frequently than the human population, allowing more rapid detection of resurgent transmission after a chemotherapy campaign.[5,6]

To obtain accurate information on the proportion of infected snails in a particular area, it is necessary to examine large numbers of snails from a variety of locations and in all seasons.[7] Snail infection is routinely monitored by examining each of the snails to see if it sheds cercariae.[8] The shedding test, however simple, does not identify snails harboring prepatent infections which may constitute a significant proportion within infected snail populations.[9] The omission of prepatent infections from snail infection rate data results in an inaccurate evaluation of transmission and interferes with the application of mathematical models of transmission.[10]

Prepatent infections in snails are currently not routinely monitored; the methods available for this purpose are not easily and accurately applicable for large numbers of snails. These methods involve microscopical examination of crushed snails for presence of larval stages,[9] or serial shedding tests over several weeks in snails transferred from the field to the laboratory.[11] In both of these cases, removal of large numbers of snails from the transmission site is required. This may

alter transmission conditions in sites containing small snail populations, and interfere with longitudinal transmission studies.[9-11] Therefore, a simple test which can identify snails infected prepatently as well as patently with human schistosomes should be useful for epidemiological studies. Such a test would have further advantages if the snails could be returned alive after testing to the site for longitudinal transmission studies.

We have developed monoclonal antibodies (Mab) which recognize, by an enzyme-linked immunosorbent assay (ELISA), *Schistosoma mansoni* antigens in the hemolymph of infected snails during prepatency and patency.[12] Only minute amounts of hemolymph were required for this purpose, and many snails were left alive after bleeding. These findings suggest the feasibility of employing Mab for the identification of snails infected with *S. mansoni* by immunodetection of schistosomal antigens. The present report provides a preliminary evaluation of the immunodetection approach with regard to test sensitivity and specificity.

MATERIALS AND METHODS

Snails and parasites

S. mansoni (Egyptian strain), originally obtained from Wellcome Laboratories, was maintained for 25 years at the Department of Parasitology, Hebrew University, in *Biomphalaria glabrata* and in mice. A Kenyan strain of *S. mansoni* was maintained in *Biomphalaria pfeifferi* and in mice at the Kenya Medical Research Institute.

Studies on laboratory snails

Normal snails (*B. glabrata*) were kept in breeding aquaria at 25°C in dechlorinated tap water. They were fed romaine lettuce and were exposed to artificial light 12 hr/day. Snails measuring 5–8 mm were infected by exposing them individually to 10 miracidia/snail. Infected snails were kept in separate containers in the dark. At peak patency, the infection rate in the *B. glabrata* colony was ≈50% as determined by shedding tests (Table 1).

The sensitivity and specificity of the immunodetection test were determined by testing snails shedding cercaria together with normal controls. For immunodetection of prepatent infections, individual snails were randomly picked from batches exposed to miracidia 1–6 weeks earlier. We also examined a group of snails infected for more than 11 weeks. A proportion of these snails had stopped shedding cercariae spontaneously; therefore, we were interested in knowing whether schistosomal antigens also disappeared under these circumstances.

Studies on field collected snails

The objective was to make snails shedding schistosome cercaria (presumably *S. mansoni*), snails shedding non-human cercariae, and snails which did not shed any cercariae at all available for the immunodetection studies. In addition, snails infected prepatently with *S. mansoni* were required for our study. It was anticipated that such snails should be found in sites where high transmission occurs.

Snails (*Biomphalaria pfeifferi*) were collected from 3 sites in Machakos District, Kenya. The sites were located in the study areas previously outlined by Siongok and others[13] and by Butterworth and others.[14] Snails were initially collected from a site on the Kakoi River[15] chosen for its remoteness from human habitation; they were therefore not expected to be infected with *S. mansoni*. Additional snails were collected from 2 separate sites on the Kinyui stream.[15] Both were proven by previous surveys to be active transmission sites of *S. mansoni*. In each case, the snails were hand collected, cleaned of debris, and shipped to a field laboratory near the village of Ilietune[14] for immediate shedding tests. Each snail was tested for cercarial shedding after exposure to daylight in individual glass vials filled with borehole water. Cercariae were examined by a magnifying glass. Snails shedding human cercariae (HC), non-human cercariae (NHC), and snails not shedding (NEG) were pooled separately and shipped to the central laboratory in Nairobi. The next day the snails were bled and their hemolymph tested for presence of schistosomal antigens.

Collection of hemolymph for immunoassay

The shell of each snail was cleaned by water and 70% ethanol using a swab and then dried. The snail was punctured near the innermost coil

of the shell and hemolymph was drawn out by a mouth operated suction tube to which a 25 G needle was attached. About 10 µl hemolymph were taken from each snail and introduced directly into wells of a microtiter plate. Each snail was bled with a separate needle. After bleeding, the hole in the shell was covered with plasticine and the snail returned to its container. Percent viability was determined immediately after bleeding and a week later. Normal safety precautions were taken when handling the snails to prevent infection.

Immunodetection

Immunodetection was carried out by ELISA.[3] Snail hemolymph (≈10 µl) was mixed with 120 ml BPS in a well of a microtiter plate and 65 µl of the mixture was then transferred to an adjoining well as a duplicate; adsorption was carried out by overnight incubation in a humid chamber. ELISA was carried out with Mab KCSme 22-3.[3] The Mab, in murine ascitic fluid, exhibited an original titer of 1:51,200 against SEA and was employed for ELISA at 1:5,000 dilution in PBS with 5% fetal calf serum. Controls, included in each plate, consisted of pooled hemolymph from infected and normal snails. These were tested with both relevant and irrelevant Mab.

RESULTS

Detection of S. mansoni *infection in laboratory snails*

The results are summarized in Table 1. Snails preselected for patency were initially tested. A total of 83 snails shedding cercariae and 117 normal control snails were included in this group. ELISA OD readings were considered positive if they were at least twice the maximal value obtained with matching normal controls. All the snails which shed cercariae gave positive results by ELISA, with an average OD of 1.075 ± 0.360 (range 0.4–1.950); normal snails were all negative, exhibiting an average ELISA OD of 0.093 ± 0.043 (range 0.00–0.193). Thus, on the average, infected snails gave ELISA OD readings which were higher than the controls by a factor of 11.5. Infected snails were differentially identified from uninfected snails with 100% sensitivity (percent ELISA positives among cercariae

TABLE 1

Detection of Schistosoma mansoni *infection in laboratory snails* (*Biomphalaria glabrata*)

Snail group	Weeks post exposure	No. snails tested	No. cercaria positive snails (% positive)	No. ELISA positive snails (% positive)	ELISA OD average ± SD	ELISA OD range	Deviation of ELISA positive from cercariae positive*
Preselected							
Infected	>5	83	83 (100%)	83 (100%)	1.075 ± 0.360	0.400–1.950	none
Normal	–	117	0	0	0.093 ± 0.043	0.000–0.193	–
Randomly picked							
At prepatency	1	25	0	0	0.078 ± 0.036	0.032–0.172	–
At prepatency	2	20	0	15 (75.0%)	0.877 ± 0.580	0.262 ± 1.841	−4.6%
At prepatency	3	25	0	21 (84.0%)	1.219 ± 0.380	0.835 ± 1.593	+4.4%
At early patency	4	16	10 (62.5%)	14 (87.5%)	0.942 ± 0.350	0.215–1.505	+17.9%
At maximal patency	5–6	59	47 (79.6%)	47 (79.6%)	1.697 ± 0.300	1.250–1.967	none
At late patency	11	26	7 (26.9%)	7 (26.9%)	1.567 ± 0.320	1.304–1.717	none

* Percent ELISA positive values of 2, 4 and 11 weeks post exposure were compared to maximal patency value (79.6%).
† ELISA OD values represent results with randomly picked snails at various times after exposure. Control OD values obtained in parallel with uninfected snails were on the average 0.090 ± 0.043 (range 0.000–0.193).

shedding snails) and specificity (percent ELISA negatives among uninfected snails). Such a complete match between ELISA positivity and cercarial shedding capacity was also evident in snails which were maintained for more than 11 weeks after exposure (Table 1). Only about a third of these snails maintained cercarial shedding capacity (25.9%) and only those shedding remained ELISA positive, suggesting that the relevant antigens disappear from the hemolymph quickly after cessation of cercarial shedding.

We then tested hemolymph taken from snails randomly picked from batches of snails exposed to miracidia 1–6 weeks earlier. As seen in Table 1, cercarial shedding was not observed 1, 2, and 3 weeks post-exposure; by 4 weeks post-exposure, patency was reached by only 62.5% of the snails. Maximal patency was reached at 5–6 weeks post-exposure, at which time only 79.5% of the exposed snails shed cercariae. By comparison, detection of infection by ELISA preceded maximal patency by about 3 weeks, as ELISA positivity was first observed at 2 weeks post-exposure. Percent ELISA positivity at this time was 75%, a little lower than maximal percent patency (79.5%). This down-deviation may be the outcome of sub-detectable amounts of the relevant antigens in the hemolymph of some of the snails during early prepatency (J. Hamburger, personal communication). By 3 and 4 weeks post-exposure, percent ELISA positivity was higher than maximal percent patency, reaching 84% and 87.5% respectively (Table 1). These results suggest that not all ELISA positive, prepatently infected snails eventually reach patency. This is in line with the existence of resistance in a proportion of the snails with the resulting destruction of parasites before onset of patency.

Immediately after bleeding for hemolymph, all snails remained alive. Snail viability a week after bleeding was 50–70%.

Detection of S. mansoni *infection in field collected snails*

These experiments were carried out in Kenya on *B. pfeifferi*. Results of initial experiments with laboratory-infected *B. pfeifferi* were similar to those obtained in the laboratory with *B. glabrata*. In both cases, a complete differentiation between infected and uninfected snails was obtained by ELISA (Table 1). Subsequently, studies on field collected snails provided an opportunity to examine the suitability of ELISA for differentially identifying snails infected with *S. mansoni* from snails infected with other trematodes.

About 500 snails were collected from a site on Kakoi River where *S. mansoni* transmission does not occur (Table 2). Of these, 60 shed non-human cercariae (NHC) and none shed schistosome cercariae. We tested by ELISA 37 which shed NHC and found them all negative. Microscopical examination of microtiter wells containing hemolymph from these snails revealed rediae and or NHC. Of the several hundred not shedding in this lot, 143 were tested by ELISA; all were found to be ELISA negative, although 33 (23%) of them exhibited rediae and or NHC by microscopical examination of the hemolymph. The non-human trematodes were identified as echinostomes or strigeids but species identification was not done. These results indicate that *B. pfeifferi* infected in nature with *S. mansoni* can be differentiated by ELISA, with 100% sensitivity and specificity, from non-infected snails and from snails infected with non-human trematodes common in the study area.

About 120 snails were collected from a known transmission site on Kinyui stream (Table 2). Of these, 8 shed schistosome cercariae and also presented schistosome cercariae or germ balls in their hemolymph. All of these 8 snails, patently infected by schistosomes, were positive by ELISA. A single case of mixed infection (*S. mansoni* with NHC) was found among the snails in this group (data not shown). This case of mixed infection was identified as positive by ELISA, indicating that the superimposed infection with non-human trematodes did not interfere with the expression of schistosomal antigens in the hemolymph during patency. Whether such interference does occur during prepatent mixed infection is not known.

A second known transmission site on Kinyui stream (near Kangundo) was characterized by a high rate of *S. mansoni* infection (6/61, or 9.8%) and by the absence of infection with non-human trematodes (Table 2). The high rate of patent infections in this site suggested the likelihood of a high rate of prepatent infections. Also, the absence of non-human trematodes precluded any possibility of mixed infections, the effect of which on the expression of schistosomal antigens in the hemolymph during prepatency is not known. Among the total of 61 snails collected in this site, 8 (13.1%) were not shedding but were ELISA positive. They represent 14.5% (8/55) of the total

TABLE 2

Detection of Schistosoma mansoni *infection in field collected snails* Biomphalaria pfeifferi

Origin of snails (features)	No. snails collected	Shedding test		ELISA			Infection rate	
		Results	No. snails	No. tested	No. negative*	No. positive*	Patent %	Prepatent by ELISA %
KEMRI (laboratory controls)	13	Neg	7	7	7	0	—	—
		Pos	6	6	0	6	—	—
Kakoi (no *S. mansoni* transmission)	~500	Neg	~440	143	143	0	0	0
		HC	0	—	—	—	—	—
		NHC	60	37	37	0		
Kinyuti Site I (transmission intermediate)	120	Neg	100	ND	ND	ND	0	ND
		HC	8	8	0	8	6.6	—
		NHC	12	12	12	0		
Kinyuti Site II (transmission high, no NHC)	61	Neg	55	55	47	8	0	13.1
		HC	6	6	0	6	9.8	—
		NHC	0	—	—	—		

* ELISA OD for negative ≤ 0.100
* ELISA OD for positive ≥ 0.300.
HC = Human Cercariae (*S. mansoni*); NHC = Non-Human Cercariae; Neg = Negative; Pos = Positive

number of those not shedding. Of these 8 snails, 4 exhibited schistosome cercariae or germ balls in the hemolymph. Judging from the high degree of specificity of the test, as seen in the preceding studies with laboratory and field collected snails, these 8 snails probably harbored prepatent *S. mansoni* infections. In this particular case, the infection rate by the shedding test alone was 9.8%, while the combined prepatent and patent infection rate (13.1 plus 9.8%) was 22.9%. These results demonstrate a case where prepatent infections constitute a substantial proportion within populations of infected snails.

DISCUSSION

We employed a Mab which recognized *S. mansoni* antigens in the hemolymph of the intermediate snail host[12] for a preliminary evaluation of the epidemiological usefulness of immunodetection by ELISA of *S. mansoni* infection in *Biomphalaria*.

Infected laboratory snails were differentiated at all stages of patency from uninfected snails with 100% sensitivity and specificity (Table 1), and the clear-cut differentiation enabled visual reading of ELISA results for qualitative determination. Studies with field collected snails (*B. pfeifferi*) demonstrated the same high degree of specificity and sensitivity in differentially detecting schistosome infected snails among a mixed population containing uninfected snails and snails infected with non-human trematodes (echinostomes and strigeids). Further evaluation of the specificity of the test should include testing of as wide a variety of non-human trematodes capable of naturally infecting *Biomphalaria* as possible. In addition, since *Biomphalaria* also serves as a natural host for *S. rodhaini*, it remains to be determined whether the test can differentiate *S. mansoni* from *S. rodhaini*. With these reservations in mind, the suitability of the Mab employed here for immunodetection of *S. mansoni* infection in field collected *Biomphalaria* is indicated. It is important to find out whether the same Mab can be employed for differential identification of *S. haematobium* in its intermediate snail host, *Bulinus*. Immunodetection can be very helpful if found suitable for species differentiation in this case, since morphological differentiation of *S. haematobium* cercariae from those of the several animal schistosomes naturally infecting *Bulinus* is impossible.

In practice, it is not expected that the immunodetection test, even if made inexpensive and usable by community health workers in endemic areas, will replace shedding tests for large-scale identification of snails patently infected with *S. mansoni*. It may, however, be useful in those areas for periodic quality control of shedding tests, in particular where routing differentiation of *S. mansoni* cercariae from a large variety of NHC is difficult. Our results with laboratory snails (Ta-

ble 1) suggest the potential of the test for identifying prepatent infections and providing more accurate snail infection rate data. Findings with field collected snails from a site with high *S. mansoni* transmission further support this suggestion by showing a case where infection rate by shedding test alone was 9.8%, whereas the combined figure of patent and prepatent infection rates was 22.9% (Table 2).

The inability of the test to identify early prepatent (1 week) infections (Table 1) is problematic in the sense that the full extent of the parasite flux from the definitive host to the snail cannot be evaluated. However, very early detection of snail infection may not be completely relevant for evaluating prospective cercarial shedding capacity, since resistance (or incompatibility) of a proportion of snails in natural populations of *Biomphalaria* may exist, resulting in destruction of the parasites at very early prepatency.[4] Since only 79.6% of the exposed laboratory snails eventually reached patency (Table 1), and assuming that practically all the young snails exposed individually to 10 miracidia/snail were penetrated by miracidia (an assumption consistent with the known high infection rates of susceptible *B. glabrata*),[16] it indeed appears that a portion of the snails destroyed the parasites sometime between miracidial penetration and maximal expression of patency. The finding that percent ELISA positivity at 3 and 4 weeks post-exposure (Table 1) is higher than maximal percent patency (at 5–6 weeks) tends to support this assumption. Moreover, loss of infection appears to be a continuous process in our system since it proceeded after maximal patency had been reached. With regard to old infections, it is important to note that ELISA positivity was proof of patency and not of residual schistosomal antigens in hemolymph after cessation of cercarial shedding.

When taken together, the results seem to suggest that percent ELISA positivity during prepatency is a more reliable estimate of prospective cercarial shedding capacity than is the miracidial penetration rate. This assumption is supported by the timing of antigen release into the hemolymph, which parallels active development and release of larvae from sporocysts.[4] Whether the amount of schistosomal antigens in the hemolymph correlates with the number of cercariae released totally or at any one point in time during patency remains to be determined.

The results justify further development of the immunoassay to provide a simple, cost-effective tool suitable for routine monitoring of prepatent *S. mansoni* infection in *Biomphalaria*. In combination with the conventional shedding test, this should provide more accurate measurements of snail infection rates and enable the determination of prepatency/patency ratios under various transmission conditions and following the application of control measures.

Another related subject which warrants attention is snail survival after bleeding for hemolymph. This subject was not addressed beyond the observation that 50–70% of the snails remained alive 1 week after bleeding. Long-term post-bleeding survival of the snails after reintroduction into the transmission site should enable the determination of the kinetics of prepatency/patency in field collected snails under various natural conditions and after application of control measures.

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REFERENCES

1. Jordan P, Webbe G, 1982. *Schistosomiasis, epidemiology, treatment, and control*. London: Heinemann, 361. UI:83054944
2. The control of Schistosomiasis. Report of a WHO Expert Committee, 1985. *WHO Tech Rep Ser* 728: 1–113. UI:86153954
3. Capron A, Dessaint JP, Capron M, Ouma JH, Butterworth AE, 1987. Immunity to schistosomes: progress toward vaccine. *Science* 238: 1065–1072. UI:88070554
4. Ouma JH, Wijers DJ, Arap Siongok TK, 1985. The effect of repeated targeted mass treatment on the prevalence of schistosomiasis mansoni and the intensity of infection in Machakos, Kenya. *Ann Trop Med Parasitol* 79: 431–436. UI:86076162
5. Sturrock RF, 1986. Snail collection to detect schistosome transmission sites. *Parasitol Today* 2: 59–63.
6. Prentice MA, Barnish G, 1981. Snail infections

following chemotherapy of Schistosoma mansoni in St. Lucia, West Indies. *Trans R Soc Trop Med Hyg 75:* 713–714. UI:82130105

7. Ansari N, Davis A, eds., 1973. *Epidemiology and control of schistosomiasis.* Baltimore: University Park Press, 752. UI:0347610
8. Chu KY, Dawood IK, 1970. Cercarial production from Biomphalaria alexandrina infected with Schistosoma mansoni. *Bull WHO 42:* 569–574. UI:70251731
9. Sturrock RF, 1973. Field studies on the transmission of Schistosoma mansoni and on the bionomics of its intermediate host, Biomphalaria glabrata, on St.Lucia, West Indies. *Int J Parasitol 3:* 175–194. UI:73183425
10. Fine PE, Lehman JS Jr, 1977. Mathematical models of schistosomiasis: report of a workshop. *Am J Trop Med Hyg 26:* 500–504. UI:77199599
11. Sturrock RF, Karamsadkar SJ, Ouma J, 1979. Schistosome infection rates in field snails: Schistosoma mansoni in Biomphalaria pfeifferi from Kenya. *Ann Trop Med Parasitol 73:* 369–375. UI:80041464
12. Hamburger J, Weil M, Anton M, Turetzky T, 1989. Snistosoma mansoni antigens recognized in Biomphalaria glabrata hemolymph by monoclonal antibodies. *Am J Trop Med Hyg 40:* 605–612.
13. Arap Siongok TK, Mahmoud AA, Ouma JH, Warren KS, Muller AS, Houser HB, 1976. Morbidity in schistosomiasis mansoni in relation to intensity of infection: study of a community in Machakos, Kenya. *Am J Trop Med Hyg 25:* 273–284. UI:76158511
14. Butterworth AE, Dalton PR, Dunne DW, Mugambi M, Ouma JH, Richardson BA, Siongok TK, Sturrock RF, 1984. Immunity after treatment of human schistosomiasis mansoni. III. Study design, pretreatment observations and the results of treatment. *Trans R Soc Trop Med Hyg 78:* 108–123. UI:84173304
15. Meuleman EA, Bayne CJ, van der Knaap WP, 1987. Immunological aspects of snail-trematode interactions. *Prog Clin Biol Res 233:* 113–127. UI:87232109
16. Anderson RM, Crombie, J, 1984. Experimental studies of age-prevalence curves for Schistosoma mansoni infections in populations of Biomphalaria glabrata. *Parasitology 89:* 79–105. UI:84296812

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Detection of *Schistosoma mansoni* DNA in extracts of whole individual snails by dot hybridization*

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The potential usefulness of DNA-DNA hybridization as a diagnostic tool has been demonstrated for a number of parasitic infections (Gonzales et al. 1984; McLaughlin et al. 1985; Pollack et al. 1985; Sim et al. 1986; Wirth and McMahon Pratt 1982) and has also been proposed for identification of schistosome infection in snails (Rollinson et al. 1986). Monitoring schistosome infection in snails is regularly required to elucidate the dynamics of transmission and the effect of control measures (Hairston 1973; Christie and Uptham 1977; Barnish 1982), both requiring examination of a large number of snails. Thus, a rapid and accurate detection method will aid parasitologists particularly since detection of prepatent infection is not straightforward and its omission leaves data on snail infection rates incomplete (Sturrock et al. 1979). Cloned DNA probes containing *S. mansoni* ribosomal RNA gene sequences were recently prepared (McCutchan et al. 1984) and used for schistosome species identification by Southern blot analysis, and for detection of infected snails by dot blot hybridization with DNA from infected snail hepatopancreas (Rollinson et al. 1986). However, any attempt to employ dot hybridization for mass screening of snail infection would require a simple procedure for treating whole individual snails for analysis. Such a procedure is described in the present report.

Snails (*Biophalaria glabrata*) were infected by exposure to 10 miracidia of *S. mansoni*. Infected and normal snails were prepared for dot hybridization. Whole snails (up to 12 mm in diameter) were each titurated in 0.3 ml 1 *M* NaOH containing 1% Triton X-100.Ituration was performed with a glass rod having a bulged and indented bottom, in a round-bottomed plastic test-tube. The titurated snails were left in the NaOH-detergent solution at room temperature for up to 4 days. Just before dot blotting, concentrated HCl was added to neutralize the samples which were then heated to 100° C for 5 min, cooled quickly, and debris removed by centrifugation at 12000 × g for 10 min. Snail extracts were individually spotted onto nitrocellulose paper (NCP), presoaked in 10 × SSC (1 × SSC is 0.15 *M* NaCl, 0.015 *M* sodium citrate) using a manifold microfiltration apparatus (Bio-rad Laboratories Richmond, CA). Conventional procedures for preparing extracts for immobilization on NCP such as proteinase K digestion and the use of SDS, resulted in an extract that was not completely filterable. Similar difficulties were encountered when the neutralization and/or heating steps were omitted.

After blotting, the NCP was washed with 10 × SSC, air-dried, baked under vacuum at 80° C for 2 h, and incubated for 4 h at 42° C in prehybridization solution containing 50% formamide, 5 × SSC, 0.05 *M* sodium phosphate pH 6.5, 75 µg/ml Salmon sperm DNA and 0.02% each of polyvinylpyrrolidone, BSA, and Ficoll. The NCP sheet was then hybridized for 10–15 h at 42° C in a solution containing 4/5 volumes of prehybridization solution, 1/5 volumes of 50% dextran sulphate, and 7 ng/ml (about 2×10^6 cpm/ml) heatdenatured ^{32}P-labelled total *S. mansoni* DNA prepared from adult worms (see below). The NCP was subsequently washed once for 20 min at room temperature with a solution containing 50% formamide, 2 × SSC, and 0.1% SDS. Then followed three washes, 20 min each at room temperature, in 2 × SSC containing 0.1% SDS, and finally two washes at 50° C for 15 min each in 0.1 × SSC containing 0.1% SDS. The NCP was air-dried and

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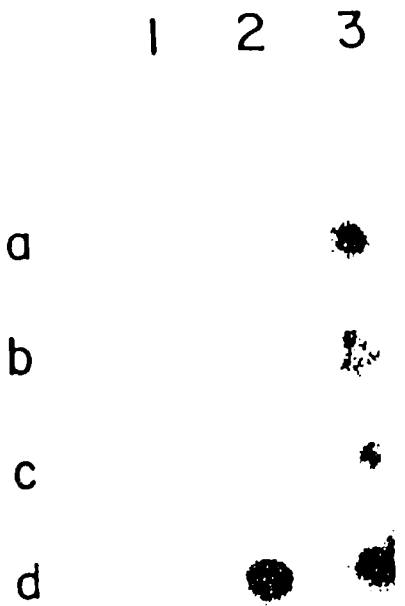

Fig. 1. Hybridization of total schistosomal DNA probe with *S. mansoni* adult worm DNA, infected snail DNA, and uninfected snail DNA. *Lane 1* Non-infected snail DNA – 1, 5, 10, 20 µg (rows *a* through *d* respectively); *Lane 2* Infected snail DNA – 1, 5, 10, 20 ng (rows a through *d* respectively); *Lane 3* Schistosomal DNA – 25, 50, 100, 200 pg (rows *a* through *d* respectively)

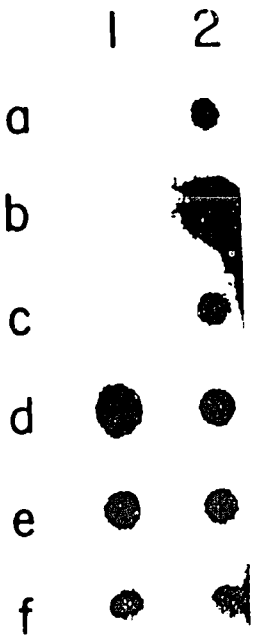

Fig. 2. Hybridization of total schistosomal DNA probe with infected and uninfected snail extracts. *Lane 1* Uninfected snails (rows *a* through *c*); Infected snails (rows *d* through *e*); *Lane 2* Infected snails (rows *a* through *e*)

autoradiographed using X-ray film (Cruix XR2, Agfa) and Cronex intensifying screen (E.I. Du Pont De Nemours Co. Inc., Wilmington, DE). Adult worm DNA, infected snail DNA, uninfected snail DNA, and whole cercariae (extracted by the procedure described above for whole snails), underwent hybridization under similar conditions.

DNA from *S. mansoni* adult worms and from infected and uninfected snails was prepared as follows. Worms (usually 1 ml packed) were washed with ice-cold TE (10 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA) and titurated by repeated passages through a syringe. Snails (usually 15) were removed from their shell, washed in TE, and cut into small pieces with a scalpel. Then they were repeatedly passed through a syringe. Five volumes of lysis buffer (10 m*M* Tris-HCl pH 8.0, 50 m*M* EDTA, 5% SDS, and 250 µg/ml proteinase K) were added and the mixture incubated for 2 h at 37° C. Phenol-chloroform extraction of DNA and alcohol precipitation were carried out by standard procedures (Maniatis et al. 1982). Alcohol-precipitated DNA pellets were vacuum-dried and treated with 100 µg/ml RNAase in TE, for 3 h at 37° C, after which the extraction and precipitation steps were repeated. Total *S. mansoni* adult worm DNA was labelled with ^{32}P-dCTP (Amersham Radiochemicals, 3000 Ci mmol^{-1}) by nick translation (Pollack et al. 1982), and used as a probe in hybridization experiments.

Figure 1 shows the results of hybridization of total DNA probe with *S. mansoni* adult worm DNA, infected snail DNA and uninfected snail DNA. As little as 25 pg *S. mansoni* DNA, and not less than 5 ng infected snail DNA gave positive signals. By comparison, about 4×10^3 times higher quantities (> 20 µg) of noninfected snail DNA did not yield positive signals. We have not tested the effect of excess snail DNA on the sensitivity of detection of schistosomal DNA. The specific signal, however, was strong enough to differentiate between infected and non-infected snail DNA.

Figure 2 shows the results of hybridization with infected and non-infected snails directly extracted and spotted on NCP without prior isolation of DNA. Non-infected snails did not show positive signals while all 9 infected snails gave positive signals.

Figure 3 shows that early prepatent as well as patent infection can be detected. In addition, intensification of positive signals can be demonstrated during the course of development of *S. mansoni* in the snail. Thus, while at 1 and 2 weeks of infection, when only mother sporocysts are present, the signal is weak. At 3 weeks, when daughter sporocysts are expected to be present, the signal

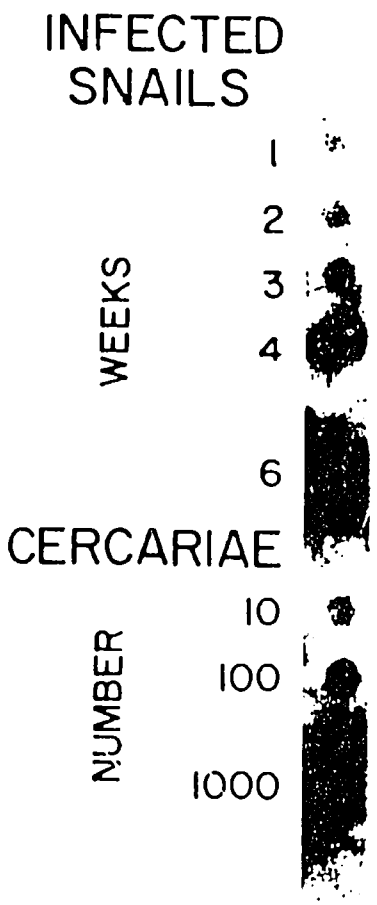

Fig. 3. Hybridization of total schistosomal DNA probe with extracts from infected snails at varying times after infection, and with cercarial extracts

strengthens and it becomes clearly stronger at 4 and 6 weeks of infection in parallel with the expected cercarial development. Extracts of 10, 100, and 1000 whole cercariae all gave signals with intensities directly correlated to cercarial numbers.

While the use of a total DNA probe appears sensitive enough for detecting infection in snails without prior laborious isolation of their DNA, it is not likely to constitute a specific tool for species differentiation in cases of mixed infections. Such differentiation was accomplished by the use of cloned *S. mansoni* ribosomal RNA gene in Southern blot analysis of different species of schistosomes. However, when used in dot hybridization it could identify infection in only some of the snails, and as much as 0.2 µg of DNA from infected snail hepatopancreas were required (Rollinson et al. 1986), suggesting a relatively low sensitivity. Moreover, since this probe gives positive signals (although with different banding) in Southern blot analysis with different schistosome species, it is not useful as a species-specific probe in dot hybridization. We have also found in preliminary experiments that a cloned *S. mansoni* DNA probe prepared from extranuclear schistosomal DNA (including mtDNA), although capable of strain differentiation in Southern blot analysis, could not detect schistosomal sequences in as much as 20 ng infected snail DNA by dot hybridization (Pollack and Hamburger 1986). Thus, it appears that a probe or a mixture of probes providing both sensitivity and specificity sufficient for mass species identification of schistosome infection in snails by dot hybridization is still unavailable.

Our present work demonstrates the feasibility of detecting patent and prepatent infections in snails by employing a simple procedure for preparing snails followed by hybridizaton with a total schistosomal DNA probe. However, several issues have to be taken into account before considering application of the approach for field epidemiology studies. Firstly, the detection of patent infection by shedding of cercariae is simple and suitable for mass screening. Secondly, the utility of the hybridization approach should be evaluated vis-à-vis crushing and microscopical examination for detecting prepatent infections. Thirdly, the degree of cross hybridization with DNA from other trematodes, commonly infecting *Biomphalaria* in nature, should be determined.

Our results suggest that quantitation of schistosomal DNA in snails may be possible by DNA hybridization followed by suitable measurements of sign intensity (Figs. 1, 3). This, however, would require standardization and calibration of the detection system. Since changes in sign intensity parallel the course of development of *S. mansoni* in the snail (Fig. 3), it may be worthwhile to evaluate the suitability of the detection approach for determining the effect of various factors (physical, chemical, and biological) on the development of *S. mansoni* in laboratory-infected snails.

References

Barnish G (1982) Evaluation of chemotherapy in the control of *Schistosoma mansoni* in Marguis Valley, Saint Lucia. Am J Trop Med Hyg 31:111–115

Christie JD, Upthari ES (1977) Control of *Schistosoma mansoni* transmission by chemotherapy in St. Lucia. II. Biological results. Am J Trop Med Hyg 26:894–898

Gonzales A, Preciger E, Huecas ME, Noguiera N, Lizard PM (1984) Microsomal repetitive DNA in *Trypanosoma cruzi*: Its use in a high sensitivity parasite detection assay. Proc Natl Acad Sci USA 81:3356–3360

Hairston NG (1973) In: Ansari N (ed) Epidemiology and control of schistosomiasis. University Park Press, Baltimore, pp 250–336

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

McCutchan TF, Simpson AJG, Mullins JA, Sher A, Nash TE, Lewis F, Richards C (1984) Differentiation of schistosomes by species, strain and sex by using cloned DNA markers. Proc Natl Acad Sci USA 81:889–893

McLaughlin GL, Edlind TD, Campbell GH, Eller RF, Ihler GM (1985) Detection of *Plasmodium falciparum* using a synthetic DNA probe. Am J Trop Med Hyg 34:837–840

Pollack Y, Hamburger J (1986) Detection of *Schistosoma mansoni* infection in snails by the use of DNA probes. Isr J Med Sci 22:851

Pollack Y, Kasir J, Shemer R, Metzger S, Szyf M (1984) Methylation pattern of mouse mitochondrial DNA. Nucleic Acids Res 12:4811–4824

Pollack Y, Metzger S, Shemer R, Landau D, Spira DT, Golenser J (1985) Detection of malaria parasites in blood using DNA hybridization. Am J Trop Med Hyg 34:663–667

Rollinson D, Walker TK, Simpson AJG (1986) The application of recombinant DNA technology to problems of helminth identification. J Parasitol 91:553–571

Sim BKL, Piessens WF, Wirth DF (1986) A DNA probe cloned in *Escherichia coli* for the identification of *Brugia malayi*. Mol Biochem Parasitol 19:117–123

Sturrock RF, Karansadkar SJ, Ouma SJ (1979) Schistosome infection rates in field snails: *Schistosoma mansoni* in *Biomphalaria pfeifferi* from Kenya. Ann Trop Med Parasitol 73:369–375

Wirth DF, McMahon Pratt D (1982) Rapid identification of *Leishmania* species by specific hybridization of kinetoplast DNA in cutaneous lesions. Proc Natl Acad Sci USA 79:6999–7003

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Highly repeated short DNA sequences in the genome of *Schistosoma mansoni* recognized by a species-specific probe

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A cloned 0.64 kb DNA sequence of *Schistosoma mansoni* contains 120-bp tandem repeats and comprises at least 12% of the schistosomal genome of both sexes. It exhibits a high degree of species specificity upon hybridization with *Schistosoma haematobium* and *Schistosoma magrebowiei* DNA, and could detect with high sensitivity schistosomal DNA sequences in infected snails.

Key words: *Schistosoma mansoni*; Repeated DNA; DNA probe; Species identification

Introduction

There are 5 schistosome species which regularly infect man. Of these, the 3 major species of human schistosomes afflict more than 200 million people around the globe in tropical and subtropical regions [1]. About a dozen other mammalian schistosomes exist in the same geographic regions, and these may infect the same freshwater snails which serve as intermediate hosts for human schistosomes. This presents a diagnostic problem, as the various schistosome species cannot be differentiated by the morphology of the larval stages developing in snails. Thus, since snail infection rates are routinely determined by identifying snails shedding schistosome larvae (cercariae) [2], it is impossible to obtain separate data on snails infected with human schistosomes in areas where human and animal schistosomes coexist. Also, since direct identification of schistosome species developing in humans is based on egg morphology alone, identification presents a problem if infection with animal schistosomes exhibiting similar egg morphology, or interspecific hybridization, is suspected [3,4]. It is therefore desirable to develop alternative means of diagnosis that will enable differentiation between species and strains of schistosomes.

Previous studies have described rDNA probes capable of differential identification of schistosome species based on restriction fragment length heterogeneity of the corresponding sequences [5,6]. Schistosome species identification was also accomplished by dot hybridization, employing as a probe a degenerate repeat present only in the genome of female *Schistosoma mansoni* [7]. In these cases the corresponding sequences are repeated approximately 100 and 75 times per haploid genome. In the present report we describe the cloning and preliminary characterization of highly repeated DNA sequences present in the genome of both male and female *S. mansoni* and exhibiting species specificity.

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Abbreviations: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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Materials and Methods

Parasites. *S. mansoni* (Egyptian strain) was originally obtained from Wellcome Laboratories about 30 years ago. It has since been maintained by passages through outbred albino mice and in snail hosts (*Biomphalaria glabrata*). Mice were infected by subcutaneous injection of 350 cercariae. Cercariae were shed under fluorescent light from snails infected 6–10 weeks previously by individual exposure to 5–10 miracidia per snail. Miracidia were induced to hatch from ova in liver homogenates of mice infected 9 weeks previously. Adult worms were collected from livers of infected mice by perfusion [8], washed once with phosphate-buffered saline (PBS), excess liquid removed, and the packed worms quickly frozen in liquid nitrogen and stored at −70°C.

Preparation of DNA. About 1 ml packed frozen worms (about 1000 worms) were thawed and washed in cold extraction buffer (50 mM Tris-HCl, pH 7.5/10 mM EDTA/100 mM NaCl), then homogenized, warmed to 50°C, supplemented with sodium dodecyl sulfate (SDS) to 100 μg ml^{-2} and incubated at 50°C for 2 h. Nucleic acids were extracted twice with phenol, once with phenol/chloroform (1:1) and once with chloroform, then dialyzed and precipitated in ethanol. Treatment with RNase A (100 μg ml^{-1}) for 3 h at 37°C then followed, and the DNA was extracted as above, dialyzed, alcohol-precipitated, dissolved in TE (10 mM Tris-HCl, pH 7.5/1 mM EDTA) and stored at −20°C. DNA of *S. haematobium* and *S. magrebowiei* was kindly supplied by Yaakov Pollack, Ben Gurion University, Beer-Sheva, Israel.

Construction of S. mansoni genomic Sau3A fragment library in the BamHI site of pUC18. DNA from *S. mansoni* adult worms was partially digested with *Sau*3A and ligated with plasmid DNA which was predigested with *Bam*HI, and dephosphorylated with calf intestinal alkaline phosphatase [9]. Several ligation reactions were carried out in parallel at various weight ratios of genomic/plasmid DNA (1.5:1, 3:1, 9:1 and 15:1), and subsequently pooled and used directly to transform competent cells of *E. coli* strain F15 by established protocols [9]. Transformed cells were plated out on LB agar plates containing ampicillin. IPTG and X-Gal were included in the final plating mixture.

Screening of library. Screening for cloned highly repeated sequences was carried out by colony hybridization. The colonies were transferred to nitrocellulose membranes, then treated with 10% SDS, and the DNA denatured in 0.5 M NaCl for 5 min and neutralized in 0.5 M Tris-HCl pH 7.5/1.5 M NaCl. Following a wash in 2 × SSPE [9] the membranes were dried and baked at 80°C for 2 h. Total genomic DNA from *S. mansoni*, ^{32}P-labeled by nick translation [10] was used as a probe. Colonies exhibiting a strong hybridization signal were selected for further screening. Plasmids from these clones were harvested by a rapid plasmid purification procedure [11], restricted with *Eco*RI and *Pst*I according to Maniatis et al. [9], then separated by agarose gel electrophoresis and underwent Southern blotting [12] and hybridization with a ^{32}P-labeled hamster rDNA probe under low stringency hybridization conditions (see below). Cloned repeated sequences unrelated to rDNA were subsequently selected.

Preparation of probes. A recombinant cosmid pH-a$_2$ [13] which contains a hamster ribosomal gene (inserted into the *Eco*RI site of the cosmid vector Mua-3) was harvested [11], restricted with *Eco*RI and fragments separated by agarose gel electrophoresis [14]. The rDNA fragment was isolated from the agarose strip by electroelution employing the model U.A.E. Unidirectional Electroeluter (International Biotechnologies, Inc. New Haven, CT, U.S.A.) according to the manufacturer's instructions. It was then labeled with ^{32}P by nick translation [10] and employed for screening to identify clones containing rDNA sequences.

A recombinant plasmid, pSm1-7, containing a 640-bp insert of *S. mansoni* DNA, comprising repeated sequences unrelated to rDNA, was selected (see below). The insert was released by digestion with *Eco*RI and *Pst*I or with *Sma*I and *Xba*I and separated by agarose gel electrophoresis and electroelution as described above. Fragments of the pSm1-7 insert were produced by *Sau*3A digestion. This was followed by electrophoresis in 5% polyacrylamide gel [14], and elution from the

polyacrylamide gel strips by diffusion in 500 mM NH_4A_c, 1 mM EDTA. DNA was subsequently extracted and dissolved in TE. The pSm1-7 insert and its fragments were ^{32}P-labeled [10] and used as probes.

Dot blot analysis. A nitrocellulose membrane was pretreated by soaking in water and washing once with 20 × SSC (1 × SSC is 0.15 M NaCl/0.015 M Na-citrate) for 30 min. DNA to be dotted was diluted as desired in 0.3 M NaCl then denatured by boiling for 10 min and kept on ice. Immediately before dotting the DNA was diluted with 2 M cold ammonium acetate then filtered through the membrane in a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, U.K.). The membrane was subsequently washed in 1 M cold ammonium acetate and dried, and the DNA underwent denaturation, neutralization and baking as described above for colony hybridization.

Filter hybridization. Standard high-stringency hybridization involved prehybridization for at least 4 h at 42°C in 50% formamide, 5 × SSPE [9], 5 × Denhardt's solution [9], 1% glycine and 200 μg ml^{-1} denaturated salmon sperm DNA. This was followed by overnight hybridization at 42°C with with heat denatured and ^{32}P-labeled probe (at least 1.5 × 10^6 cpm ml^{-1}) in hybidization solution consisting of 50% formamide, 5 × SSPE, 1 × Denhardt's solution, 0.3% SDS and 120 μg ml^{-1} denatured salmon sperm DNA. Hybridized membranes were washed twice with each of the following solutions; 2 × SSPE for 5 min at room temperature (RT); 2 × SSPE/0.1% SDS for 30 min at 53°C; 0.5 × SSPE/0.1% SDS for 30 min at 53°C, 0.1 SSPE/0.1% SDS for 30 min at 53°C; and finally with 2 × SSPE at RT for 5 min. The membranes were then airdried and subjected to autoradiography (see below). Densitometry of dot blot autoradiograms was carried out using the 'Quick-Scan R&D Densitometer' (Helena Laboratories, Beaumont, TX, U.S.A.). Hybridization at low stringency with the ^{32}P-labeled hamster rDNA probe p11-a2 [13] involved prehybridization in 1 × Denhardt's solution for 4 h at 60°C. This was followed by hybridization at 37°C with the heat-denatured radioactive rDNA probe in 1.0 M NaCl/10 Tris-HCl, pH 7.4, 10 × Denhardt's solution and 10% formamide. The membrane was subsequently washed five times each for 30 min at 42°C with 6 × SSC (1 × SSC is 0.15 M NaCl/0.015 M Na-citrate), air-dried, and subjected to autoradiography using a Kodak X-ray film, and a 'Carnex' intensifying screen.

DNA sequencing. The nucleotide sequence of the schistosomal DNA inserted in pSm1-7 was determined using Sanger's dideoxy chain termination technique [15] as modified by Hattori and Sakaki [16] for double-stranded sequencing, using denatured plasmid template. The 'Sequenase' kit (Stratagene, La Jolla, CA) and ^{35}S-labeled dATP were employed.

Results

Identification of clones containing species specific DNA sequences unrelated to rDNA. About 5000 recombinant colonies representing a partial genomic library of *Sau*3A-digested *S. mansoni* DNA in the *Bam*HI site of pUC18 underwent hybridization with total ^{32}P-labeled genomic DNA of *S. mansoni*. A total of 140 colonies exhibiting strong hybridization signals were identified, amplified and cryopreserved. Each of these clones was digested with *Eco*RI and *Pst*I to release the inserted *S. mansoni* DNA fragments and underwent agarose gel electrophoresis, Southern blotting and hybridization with ^{32}P-labeled hamster rDNA isolated from p11-a$_2$ [13]. About 40 clones which did not react with the hamster rDNA were selected. A preliminary study on species specificity of these 40 clones was carried out by colony hybridization with ^{32}P-labeled DNA from *S. mansoni*, *S. haematobium* and *S. magrebowiei*. Five clones exhibiting strong hybridization signals with *S. mansoni* DNA, and none with *S. haematobium* or *S. magrebowiei* were identified, and shown to cross-hybridize with each other. One of these clones, pSm1-7, containing a 640-bp insert of *S. mansoni* DNA was taken for further analysis.

Abundance and arrangement of the repeated sequence. Preliminary information on the abundance of the repeat was obtained from quantitative dot-blot analysis. Equal quantities of ^{32}P-labeled pSm1-7 insert were hybridized with various con-

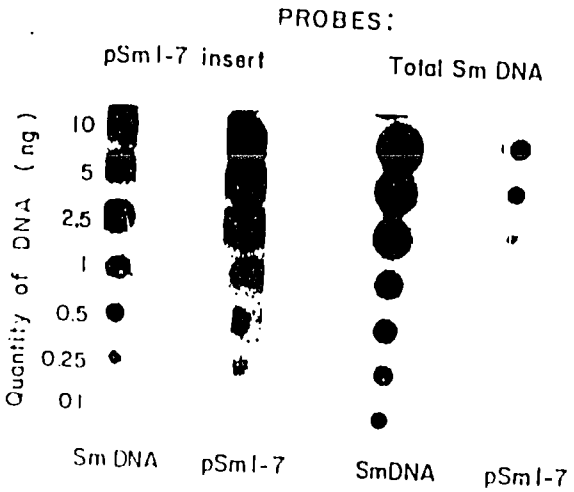

Fig. 1. Autoradiogram of different quantities of total *S. mansoni* DNA (SmDNA) and of pSm1-7, dot-blotted and then hybridized with ^{32}P-labeled pSm1-7 insert or with total SmDNA.

centrations of total *S. mansoni* DNA and pSm1-7 recombinant plasmid DNA dotted on NCP (Fig. 1). Similar hybridization signals were obtained in both cases with DNA quantities ranging between 100 pg and 1 ng. This similarity is further demonstrated in Fig. 2 which presents the results of densitometric analysis for determining the relation between DNA quantity and intensity of the hybridization signal. Given the similarity of the

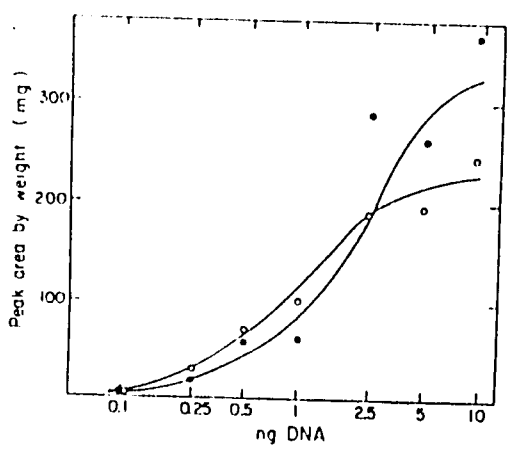

Fig. 2. Graphic representation of the intensity of hybridization signals obtained with SmDNA (;cT) and pSm1-7 (•) upon probing with pSm1-7 insert. The corresponding hybridization dots (see Fig. 1) were analyzed by densitometry, and peak integrals determined by weighing the paper underneath each peak.

hybridization signals as seen in the lower section of the curve (DNA quantities ranging between 100 pg and 1 ng) and since the schistosomal insert DNA constitutes only about 1/6 of total pSm1-7 DNA it can be estimated that the insert represents about 17% of the total schistosomal genome. In a reciprocal experiment total ^{32}P-labeled *S. mansoni* DNA was used as a probe in a quantitative dot hybridization with total *S. mansoni* DNA and pSm1-7 (Fig. 1). The signals obtained with 5 ng and 10 ng pSm1-7 were roughly similar by visual examination to 0.1 ng and 0.25 ng total *S. mansoni* DNA, respectively (Fig. 1). The visual similarity corresponds to at least 80% similarity by densitometric analysis (not shown). Calculation of the ratio of signal strength (by weight of peak area on paper) to DNA quantity gave average values of 234.8 for 0.1 ng and 0.25 ng total *S. mansoni* DNA and 4.6 for 5 ng and 10 ng pSm1-7. The value for pSm1-7 is thus 1.96% of the total DNA. However, considering that the schistosomal 0.64-kb sequence constitutes about 1/6 of pSm1-7, it can be estimated that this pSm1-7 insert constitutes about 12% of the schistosomal genome.

DNA from *S. mansoni* males and females and from mixed sexes was partially digested with *Sau*3A, and underwent electrophoresis and Southern blot analysis [12] with pSm1-7 schistosomal DNA insert. A ladder of bands was observed (Fig. 3), each band varying in length by about 120 bp. This pattern indicates that the schistosomal DNA contains a tandemly repeated sequence about 120 bp long, and that this sequence is present in pSm1-7. This tandem repeat is present in both female and male *S. mansoni* adult worms. Partial sequence analysis of pSm1-7 insert revealed the presence of a dimer of a 120 bp sequence and a start of a third such repeat. This sequence is presented in Fig. 4. A complete *Sau*3A digestion of the 640-bp insert yielded fragments 240 bp, 120 bp and 80 bp long (Fig. 5). These fragments were isolated, labeled with ^{32}P and used as probes at similar radioactivity/size ratios in dot blot hybridization against various quantities of total *S. mansoni* DNA. The pSm1-7 insert fragments exhibited similar detection sensitivities, namely between 100 and 250 pg (Fig. 6). These results indicate that all parts of the pSm1-7 insert are equally highly represented in the schistosomal genome.

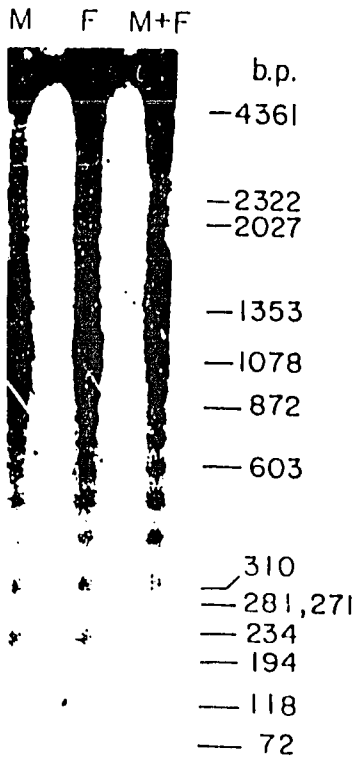

Fig. 3. Southern blot analysis of *S. mansoni* DNA (M, adult males; F, females; F + M mixed sexes) digested with *Sau*3A and probed with ^{32}P-labeled pSm1-7 insert. The position of molecular weight markers is indicated.

Fig. 4. *S. mansoni* 120-bp repeat sequence.

Upon dot-blot hybridization (results not shown) the 120 bp fragment hybridized with both the 240-bp and the 80-bp fragments, suggesting that the 640-bp insert in pSm1-7 is composed entirely of sequences closely homologous to the 120-bp repeat units. The intensity of staining of individual bands (Fig. 5) suggest that the 640-bp insert is composed of one 240-bp fragment, two 120-bp fragments and one 80-bp fragment. The 80-bp unaccounted for may be the result of generation of very small fragments. Minor changes in nucleotide sequence may be present in the distal, yet

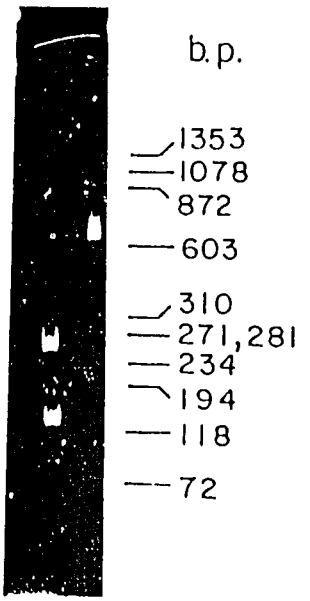

Fig. 5. Polyacrylamide gel electrophoresis of the pSm1-7 insert (640-bp) following digestion with *Sau*3A.

Fig. 6. Autoradiogram of quantitative dot blot hybridization of total *S. mansoni* DNA (SmDNA) upon probing with ^{32}P-labeled fragments (240-bp, 120-bp and 80-bp) of the schistosomal DNA insert in pSm1-7.

unsequenced, part of the 640-bp sequence to create additional *Sau*3A restriction sites that may account for the generation of very small fragments.

Sensitivity and specificity of the pSm1-7 insert for detecting schistosomal DNA sequences. Analysis by quantitative dot hybridization demonstrated

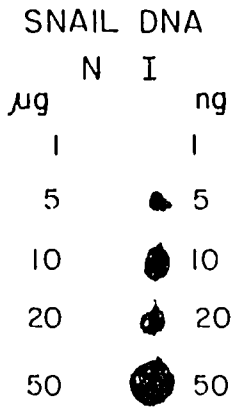

Fig. 7. Detection of *S. mansoni* DNA sequences in DNA from infected snails (I) and from normal controls (N) by dot blot hybridization with ^{32}P-labeled pSm1-7 insert.

that ^{32}P-labeled pSm1-7 insert can detect as little as 100 pg schistosomal DNA (Fig. 1). The pSm1-7 insert was then used as a probe to detect schistosomal DNA sequences in infected snails by dot hybridization (Fig. 7). This probe revealed schistosomal sequences in as little as 1 ng DNA from infected snails, while as much as 50 μg of normal snail DNA gave negative results.

Clone pSm1-7 was originally selected for species specificity by colony hybridization with total ^{32}P-labeled DNA from *S. mansoni*, *S. haematobium* and *S. magrebowiei*. Quantitative dot hybridization was subsequently carried out. *S. haematobium* and *S. magrebowiei* DNA (1–1000 ng), and *S. mansoni* (1–5 ng) were dotted on NCF and probed with ^{32}P-labeled pSm1-7 insert (Fig. 8). *S. haematobium* DNA at 500 ng and 1000 ng gave positive signals, but none was detected with any of *S. magrebowiei* DNA quantities tested. Judging from the maximal sensitivity (100 pg) of *S. mansoni* DNA detection by pSm1-7 insert (Fig. 1) it appears that the *S. mansoni* specificity of the pSm1-7 insert is >1000 vis à vis *S. haematobium*, and >>1000 vis à vis *S. magrebowiei*. Since only two heterologous schistosome species were available for the test, the evidence for the species specificity of the pSm1-7 insert is considered partial.

Discussion

We have cloned and characterized a short, tandemly repeated DNA sequence which is highly

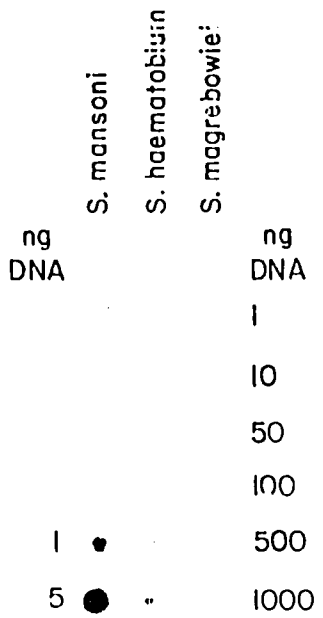

Fig. 8. Determination of the species specificity of the pSm1-7 probe. Different quantities of DNA from *S. mansoni* (1 and and 5 ng) and from the heterologous species *S. haematobium* and *S. magrebowiei* (1–1000 ng) were dotted and then probed with the ^{32}P-labeled pSm1-7 insert.

represented in the genomes of both male and female *S. mansoni*. Repeated DNA sequences have been used by other investigators as probes for detecting parasites and for identifying their taxon [15,17,18,19]. Small, highly repeated sequences can be considered highly suitable probes for such purposes. Firstly, because their high representation obviates a high detection sensitivity, and secondly, because such sequences are most likely non-coding and therefore evolve more rapidly than the rest of the genome. Species identification of schistosomes has so far been accomplished by employing ribosomal gene probes in Southern blot analysis to demonstrate species specific restriction fragments [5]. However, such a test cannot be considered suitable for multiple assays where simplicity and low cost are crucial. Also since the ribosomal gene is represented only in about 100 copies/haploid genome [6], the detection sensitivity of the rDNA probe is relatively low.

A DNA probe from *S. mansoni* which represents a tandemly repeated 0.4-kb degenerative sequence, present approximately at 75 copies per adult female genome [7] was recently described.

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This probe allows determination of sex in larval parasites. It is specie-specific vis à vis a number of schistosome species tested allowing species identification by simple dot hybridization, but the degree of specificity has not been addressed. Clone pSm1-7 described here contains a 0.64-kb schistosomal sequence which constitutes at least 12% of the total *S. mansoni* genome. Further analysis with a DNA probe of known size and copy number is required in order to exactly determine the abundance of the cloned pSm1-7 repeat sequence within the schistosomal genome. However, the results so far (Fig. 1) clearly indicate its high representation and correspondingly high detection sensitivity. A repetitive sequence of such a high representation has not been yet described in the schistosomal genome. Whether this may be ascribed to strain differences remains to be examined. Analysis of the species specificity of pSm1-7 is yet only partial, but the degree of specificity detected with *S. mansoni* is at least 3 orders of magnitude higher than with *S. haematobium* and *S. magrebowiei*. Elucidation of the full differentiation potential of this probe requires quantitative dot blot analysis with a wide spectrum of schistosome species and strains. The cross-reactivity demonstrated with *S. haematobium* (Fig. 8) suggests that a similar repeat is present in *S. haematobium*. It is therefore expected that the pSm1-7 probe should be suitable for identifying partially cross-hybridizing sequences from the heterologous schistosome species (under suitable hybridization conditions). From a practical point of view the differential identification of *S. haematobium* from other schistosomes developing in *Bulinus* should provide more accurate snail infection rate data, and may also enable differentiation between species of terminal-spined schistosomes developing in the vertebrate. Comparative sequence analysis may be helpful for elucidating evolutionary relations between schistosome species and strains, and for constructing highly specific oligonucleotide probes as has been demonstrated for filariae [19].

The pSm1-7 probe could detect schistosomal DNA in 1 ng infected snail DNA (Fig. 7) confirming the high detection sensitivity and indicating that the repeats are highly represented in DNA from *S. mansoni* intramolluscan larvae. The high detection sensitivity should allow rapid detection of individual infected snails without prior purification of DNA from the snails. A procedure for this purpose was previously described by us [20].

Acknowledgements

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References

1 World Health Organization (1985) The control of Schistosomiasis. Technical Report Series 728.4. WHO, Geneva.
2 Jordan, P. and Webbe, G. (1982) Schistosomiasis: Epidemiology, Treatment and Control. Heinemann, London.
3 Pitchford, R.J. (1959) Cattle schistosomiasis in man in the eastern Transvaal. Trans. R. Soc. Trop. Med. Hyg. 55, 285–290.
4 Wright, C.A. and Southgate, V.R. (1976) Hybridization of schistosomes and some of its implications. In: Genetic Aspects of Host-Parasite Relationships (Symp. Br. Soc. Parasitol. 14) (Taylor, A.E.R. and Muller, R. eds.), pp. 55–86, Blackwell, Oxford.
5 McCutchan, T.F., Simpson, J.G., Mullins, J.A., Sher, A., Nash, T.E., Lewis, F. and Richards, C. (1984) Differentiation of schistosomes by species, strain and sex by using cloned DNA markers. Proc. Natl. Acad. Sci. USA 81, 889–893.
6 Rollinson, D., Walker, T.K. and Simpson, A.J.G. (1986) The application of recombinant DNA technology to problems of helminth identification. Parasitology 91, 853–854.
7 Walker, T.K., Rollinson, D. and Simpson, J.G. (1989) A DNA probe from *Schistosoma mansoni* allows rapid determination of the sex of larval parasites. Mol. Biochem. Parasitol. 33, 33–100.
8 Duvall, R.N. and De Witt, W.B. (1976) An improved perfusion technique for recovering adult schistosomes from laboratory animals. Am. J. Trop. Med. Hyg. 16, 483–486.
9 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
10 Rigby, P.W.J., Dieckman, M., Rhodes, C. and Berg, P. (1977) Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase 1. J. Mol. Biol. 113, 237–251.
11 Ish-Horowicz, D. and Burke, J. (1981) Rapid and efficient cosmid cloning. Nucleic Acids Res. 9, 2989–2998.
12 Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503–517.
13 Wahl, G.M., Vitto, L. and Rubniz, J. (1983) Coamplification of rRNA genes with CAD genes in *N*-(phosphoacetyl)-L-aspartate-resistant syrian hamster cells. Mol. Cel. Biol. 3, 2066–2075.
14 Sealy, P.G. and Southern, E.M. (1984) In: Gel Elec-

trophoresis of Nucleic Acids (Rickwood, D. and Homes, B.D., eds.), pp. 39–76. IRL Press, Washington, DC.

15 Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5468.

16 Hattori, M. and Sakaki, Y. (1986) Dideoxy sequencing method using denaturated plasmid templates. Anal. Biochem. 152, 232–238.

17 Wirth, D.F. and Pratt, D.M. (1982) Rapid identification of Leishmania species by specific hybridization of kinetoplast DNA in cutaneous lesions. Proc. Natl. Acad. sci. USA 79, 6999–7003.

18 Gonzales, A., Prediger, E., Huecas, M.E., Nogueira, N. and Lizardi, P.M. (1984) Minichromosomal repetitive DNA in *Trypanosoma cruzi* its use in a high-sensitive parasite detection assay. Proc. Natl. Acad. Sci. USA 81, 3356–3360.

19 Piessens, W.F., McReynolds, L.A. and Williams, S.A. (1987) Highly repeated DNA sequences as species specific probes for Brugia. Parasitol. Today 3, 387–389.

20 Hamburger, J., Weil, M. and Pollack, Y. (1987) Detection of *Schistosoma mansoni* DNA in extracts of whole individual snails by dot hybridization. Parasitol. Res. 74, 92–100.