Schistosoma mansoni Cercaria and Schistosomulum Antigens in Serodiagnosis of Schistosomiasis[1]

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Schistosoma mansoni *cercaria and schistosomulum obtained* in vitro *were used in immunofluorescence (IF) tests and indirect hemagglutination (IHA) tests of 137 study sera, 44 from subjects infected with* S. mansoni *and 93 from healthy subjects residing outside areas endemic for the disease. The results of these tests were compared with those obtained by testing the same sera using conventional adult worm antigen, and also with the initial clinical and parasitologic diagnoses of the 137 subjects providing the study sera.*

Regarding sera from acute versus chronic cases, IF testing of the acute sera consistently detected IgA antibodies along with IgM and IgG, the last two being found consistently in chronic sera. Also, the geometric mean of the IgM antibody titers found in the IF tests was higher for acute than for chronic sera.

Excluding IF IgA, which was negative for chronic cases, the sensitivity of the other types of tests (IF IgG, IF IgM, and IHA) using cercaria and schistosomulum antigens, both under evaluation, ranged from 0.773 to 0.955, the specificity ranged from 0.957 to 1.000, the efficiency ranged from 0.927 to 0.985, the predictive value of positives ranged from 0.909 to 1.000, and the predictive value of negatives ranged from 0.903 to 0.979. No statistical differences were observed between these results and those obtained with conventional adult worm antigen. This suggests that cercaria and schistosomulum antigens, both of which can be produced more quickly and cheaply than adult worm antigen, could serve as reliable alternatives to adult worm antigen in the serodiagnosis of schistosomiasis mansoni.

In areas endemic for schistosomiasis caused by *Schistosoma mansoni* (known as schistosomiasis mansoni), diagnostic techniques involving direct observation of the parasite (eggs in stools) are generally preferred. However, misleading diagnoses are frequent among patients having infections of low to moderate intensity, unisexual infections, and other sorts of infections in which factors such as the advanced age of the patient or lateness in the stage of the parasites' life cycle considerably diminish the number of *S. mansoni* eggs in the patient's feces (*1, 2*).

Within this frame of reference, serologic assays have proven valuable in many different ways—by disclosing infections missed by parasitologic techniques, estimating the prevalence of infection in untreated populations, determining the incidence of infection in communities covered by control programs, and, to some

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extent, in monitoring patient chemotherapy (*3–5*). At present an array of serologic tests is available (*2*). These generally employ adult worm or parasite egg antigens (either crude or pure); and while the potential has existed for use of other antigen sources, up to now the advantages involved have seemed uncertain.

Cercaria antigen was used in immunofluorescence (IF) testing during the late 1960s and early 1970s. However, until recently no further research was done to overcome this procedure's low specificity and other features making it impractical for use in seroepidemiologic surveys (*6*). More recently, soluble cercarial extracts were used in indirect hemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) serology (*7*), but their diagnostic utility was not ascertained.

The diagnostic potential of antigen derived from schistosomulum, the larval stage of the parasite that develops after skin penetration (obtained either *in vivo* or through *in vitro* cultivation), has not been assessed before, although antibodies against outer membrane schistosomulum have been found in sera from patients with schistosomiasis mansoni (*8*).

Antigens from these early stages in the parasite's development offer certain advantages over antigens from the adult stages. Specifically, they can be obtained more quickly, easily, and cheaply than adult antigens because they require less time to develop and no expensive facilities for maintaining vertebrate hosts—points of particular interest to developing countries.

With this in mind, we sought to determine whether antigens from cercaria and schistosomulum cultivated *in vitro* could be used effectively to detect IgG, IgM, and IgA antibodies separately in IF tests and to detect total antibodies (class unspecified) in IHA tests. The sera examined in these tests were obtained from patients with various different clinical forms of schistosomiasis mansoni. The results obtained with the cercaria and schistosomulum antigens were compared to results obtained by testing the same sera with conventional IF and IHA procedures employing adult worm antigen.

MATERIALS AND METHODS

Serum Samples

A total of 137 serum samples were studied. Forty-four of these came from patients with schistosomiasis mansoni who had yielded positive parasitologic results when tested by the Kato-Katz method (*9*). Of the 44 cases, 10 were classified as acute and 34 as chronic, the latter including 14 infections considered intestinal, 10 hepatointestinal, and 10 hepatosplenic according to Neves' criteria (*10*) for clinical forms of schistosomiasis. The remaining 93 sera were obtained from clinically healthy individuals whose stool examinations yielded negative results. These people included 60 residents of the municipality of São Paulo and 33 residents of the municipality of Iguape, both of these being areas that are nonendemic for schistosomiasis mansoni. All of the serum samples were divided into 1 ml aliquots and stored with glycerin at −20°C (*11*).

Antigens

S. mansoni cercariae (LE strain, Belo Horizonte) were collected from specimens of the intermediate snail host *Biomphalaria glabrata* 40 days after infection. They were then concentrated in glass conical centrifuge tubes at 4°C as described previously (*12*). This cercaria suspension was divided into two parts, both of which were centrifuged. In one case the resulting sediment was embedded in a gel (Tissue-Teck OCT Ames Co., Miles

Laboratories, U.S.A.), frozen, and sectioned in a cryostat for use in the IF test. In the other the sediment was stored at −20°C until used as antigen for the IHA test (*13*).

Schistosomula cultivated *in vitro* were obtained as previously described (*14*). In brief, the cercaria suspension was centrifuged and resuspended in Earle's medium[4] enriched with lactalbumin (ELAC). Cercaria bodies were prepared by stirring the suspension for 60 seconds in a Vortex Jr. homogenizer (Scientific Industries, Inc., Queens Village, New York, U.S.A.). After allowing this suspension to settle for 10 minutes, the tail-rich supernatant was discarded, and the sedimented bodies were resuspended in ELAC medium. This washing procedure was repeated two or three times, after which the cercaria bodies in ELAC medium were incubated for two hours at 37°C. The schistosomula obtained in this manner were then divided into two portions for preparation of IF and IHA antigens using the procedure described above for cercaria.

Adult worms were processed in a similar manner (*12*) to provide IF and IHA antigens.

Serologic Tests

The cryostat sections of cercaria, schistosomulum, and adult worm concentrates were assayed in IF tests with anti-human IgG (γ-chain), anti-human IgM (μ-chain), and anti-human IgA (α-chain) fluorescent conjugates supplied commercially (Hyland Div. Travenol Lab., U.S.A.), after checking the latter's monospecificity by immunoelectrophoresis.

[4]Earle's balanced salt solution containing 0.68% NaCl, 0.04% KCl, 0.0125% $NaH_2PO_4H_2O$, 0.02% $CaCl_2$, 0.01% $MgSO_4$, 0.22% $NaHCO_3$, 0.005% phenol red, and 0.1% glucose.

Before attempting to detect IgM antibodies, the rheumatoid factor was removed by absorption of sera with human gamma globulin aggregates (*15*).

For the IHA test, formalin-stabilized human group O red blood cells were coated with alkaline-solubilized cercaria, schistosomulum, and adult worm preparations (*13*).

Statistical Analyses

Each serologic test using different *S. mansoni* antigens was evaluated with respect to its sensitivity, specificity, efficiency, and the predictive values of positive and negative results (*16*). The kappa (κ) index of agreement between each test and the true (clinical/parasitologic) diagnosis was determined. In addition, the kappa index of agreement was determined for each test using cercaria or schistosomulum antigen with respect to the corresponding reference test using adult worm antigen. The McNemar test (*17*) was applied to determine whether the positive and negative results of the tests under investigation differed significantly from those of the reference test or the true diagnosis. Student's t test was used to compare the geometric mean IgM and IgG antibody titers (GMT) detected in acute infections with the GMT found in chronic infections (*18*).

RESULTS AND DISCUSSION

The work reported here was directed at investigating the validity of using cercaria and schistosomulum antigens for IF and IHA tests by comparing the results obtained with the results of reference tests using adult worm antigen and also with the true diagnostic results based on clinical and parasitologic data. The investigation appears relevant, since neither the use of early *S. mansoni* developmental stages nor the types of antigen prepara-

tions employed (notably cryostat sections for the IF tests and alkaline-solubilized extracts for the IHA tests) have been thoroughly explored.

The IF tests of the 10 acute case sera that used cercaria and schistosomulum antigens succeeded in detecting IgG, IgM, and IgA antibodies in a fashion similar to reference tests using adult worm antigen. The former antigens caused fluorescent staining of the parasite membrane and, to a lesser extent, the parenchyma. In tests with conventional adult worm antigen the fluorescence was limited to the gut and occasionally the membrane.

The IF tests of sera from chronic cases using the two study (cercaria and schistosomulum) antigens detected IgG and IgM antibodies that yielded fluorescence patterns similar to those observed with the acute sera. The IF tests of chronic sera with adult worm antigen also detected IgG and IgM antibodies, the IgG strongly staining the parasite parenchyma while the IgM only reacted with the gut.

In general, the IgA and IgM antibodies detected with the cercaria and schistosomulum antigens produced good fluorescent staining, easily visualized in the microscope's dark field. This was clearly superior to the fluorescent staining of the gut obtained with the adult worm antigen, which was sometimes weak and was not present in all sections.

The cutoff titer established for each serologic test was selected so as to yield the highest diagnostic efficiency in discriminating between individuals who were and were not infected with *S. mansoni*. Thus, for IF tests using the cercaria and schistosomulum antigens, the cutoff titer was 80 for IgG but 10 for both IgM and IgA. For IHA tests using the same antigens, the cutoff titer was 20. For the conventional IF tests using adult worm antigen, the cutoff titer was 20 for IgG but 10 for IgM and IgA. And for the conventional IHA tests using adult worm antigen, the cutoff titer was 20.

The values indicated by the data for sensitivity, specificity, and efficiency, as well as the predictive values of positives (PV+) and negatives (PV−), are shown in Table 1. The values found for all the serologic tests using the cercaria and

Table 1. Diagnostic performance of IgG, IgM, and IgA immunofluorescence (IF) tests and indirect hemagglutination (IHA) tests using different *S. mansoni* antigens in the study of 137 patients with schistosomiasis mansoni and uninfected individuals.

Test	Antigen	Sensitivity	Specificity	Efficiency	PV+	PV−
IgG IF	Cercaria	0.909	0.957	0.942	0.909	0.957
	Schistosomulum	0.955	1.000	0.895	1.000	0.979
	Adult worm	1.000	1.000	1.000	1.000	1.000
IgM IF	Cercaria	0.955	1.000	0.985	1.000	0.979
	Schistosomulum	0.864	1.000	0.956	1.000	0.939
	Adult worm	0.977	1.000	0.993	1.000	0.989
IgA IF	Cercaria	0.227	1.000	0.752	1.000	0.732
	Schistosomulum	0.227	1.000	0.752	1.000	0.732
	Adult worm	0.295	1.000	0.774	1.000	0.750
IHA	Cercaria	0.886	1.000	0.964	1.000	0.949
	Schistosomulum	0.773	1.000	0.927	1.000	0.903
	Adult worm	0.977	0.892	0.920	0.811	0.988

Note· Sensitivity = true positives detected/(true positives + false negatives); specificity = true negatives detected/(true negatives + false positives); efficiency = (true positives detected + true negatives detected)/total number tested; predictive value of positives (PV+) = true positives detected/(true positives + false positives); and predictive value of negatives (PV−) = true negatives detected/(true negatives + false negatives).

schistosomulum antigens are close to those provided by reference tests using adult worm antigen. Within this context, it is worth noting that the sensitivity and specificity values obtained in the IgG IF test with the cercaria antigen employed here are higher than those values reported from tests carried out with the whole cercaria body (*6*).

As can be seen, the sensitivity of the IgA IF test with all three antigens was relatively low because most of the sera tested were obtained from patients with chronic cases. In general, our past research (*12*) as well as the work reported here has shown a positive correlation between the presence of IgA antibodies and acute schistosomiasis infection. Thus, IgA antibodies can be considered better immunologic indicators of acute schistosomiasis than IgM antibodies because they are detected at higher titers in acute infections than in chronic infections. It is true that the present study did detect IgA antibodies in five serum samples from patients with chronic infections, but the positive titers involved were relatively low, ranging from 20 to 40. IgA antibody in four of these samples reacted only with adult worm antigen, while that in the remaining sample reacted with both cercaria and schistosomulum antigens but not with adult worm antigen. In contrast, IgA antibody in samples from patients with acute cases invariably reacted with all three types of antigens, yielding titers ranging from 10 to 640 with adult worm antigen and from 40 to 640 with cercaria and schistosomulum antigens (see Table 4). Because study of other unrelated disease has associated the presence of IgA antibodies with mucosal involvement (*19*), it seems likely that the small number of chronic patients testing positive by IgA IF might have had lesions of the intestinal mucosa.

Although other investigators (*20, 21*) reported some time ago that IgA antibodies were present in sera from patients with undefined clinical forms of schistosomiasis, the finding that IgA antibodies are present in sera from patients with acute infections was only recently confirmed (*22*).

It has been determined that the observed IgA antibodies are reactive against worm gut, cercaria, and schistosomulum membranes, and that they probably share the same reported (*23*) carbohydrate epitopes of IgM and IgG antibodies, both of which are observed in sera from acute infections. Since these carbohydrates from schistosomulum antigens cross-react with keyhole limpet hemocyanin (*24, 25*), a search for cross-reactivity with other bacterial polysaccharide antigens seems relevant.

Table 2 shows the kappa (κ) agreement index, as well as the degree of κ agreement, between the diagnosis indicated by each variety of test and the true diagnosis. All the κ indices calculated for tests using cercaria and schistosomulum antigens were similar to those calculated for the reference tests using adult worm antigen. In addition, all the tests except the IgA IF tests had statistically significant κ indices of agreement that were rated as "almost perfect."

Table 3 shows the κ agreement index and degree of κ agreement between the test using cercaria and schistosomulum antigens and the corresponding reference tests using adult worm antigen. All the IF tests using cercaria and schistosomulum antigens exhibited high κ indices of agreement rated as "almost perfect," while the IHA tests yielded slightly lower κ indices of agreement rated as "substantial." All the κ indices obtained in this comparison were statistically significant.

Table 4 shows the numbers of test sera obtained from normal subjects and from patients with different clinical forms of schistosomiasis mansoni, and also shows

Table 2. The degree of kappa (κ) index agreement between (a) the test results using cercaria, schistosomulum, and adult worm antigens and (b) the true (clinical and parasitologic) diagnoses of the subjects providing the 137 test sera.

Antigen	Test	Kappa index (κ)	Strength of κ	Z_o[a]
Cercaria	IgG IF	0.866	Almost perfect	5.58
	IgM IF	0.950	Almost perfect	6.04
	IgA IF	0.271	Slight	1.30
	IHA	0.897	Almost perfect	5.58
Schistosomulum	IgG IF	0.950	Almost perfect	6.04
	IgM IF	0.879	Almost perfect	5.40
	IgA IF	0.271	Slight	1.30
	IHA	0.806	Almost perfect	5.58
Adult worm	IgG IF	1.000	Almost perfect	6.44
	IgM IF	0.983	Almost perfect	6.27
	IgA IF	0.363	Slight	1.78
	IHA	0.825	Almost perfect	5.78

[a] Z_o = observed Z (critical Z = 1.96 for 0.05 level). See references *26–28*.

the numbers of sera in each group testing positive in each type of test performed. Within each variety of test, each of the three antigens yielded positive and negative results that did not differ significantly from those obtained with the two other antigens. (When the McNemar test was applied, the χ^2 obtained were all lower than the critical χ^2 = 1.36, degrees of freedom (d.f.) = 1, at the 0.05 level.)

The overall analysis indicated that the geometric mean titers (GMT) of the IgM antibodies detected in sera from acute cases were significantly higher than those detected in sera from chronic cases, irrespective of the type of antigen used.

Table 3. The degree of kappa (κ) index agreement between (a) the test results using cercaria and schistosomulum antigens and (b) the test results using adult worm antigen.

Antigen	Test	Kappa index (κ)	Strength of κ	Z_o[a]
Cercaria	IgG IF	0.866	Almost perfect	5.58
	IgM IF	0.966	Almost perfect	6.09
	IgA IF	0.818	Almost perfect	2.19
	IHA	0.759	Substantial	5.15
Schistosomulum	IgG IF	0.950	Almost perfect	6.04
	IgM IF	0.878	Almost perfect	5.29
	IgA IF	0.818	Almost perfect	2.19
	IHA	0.655	Substantial	4.25

Note: The observed Z for IgA antibodies were lower than the observed Z for other antibody isotypes because the proportion of IgA positive results was low in relation to the total number of sera studied. Since the ability of cercaria and schistosomulum antigens to detect IgA antibodies was similar to that of worm antigen, the κ indices were higher.

[a] Z_o = observed Z (critical Z = 1.96 for 0.05 level). See references *26–28*.

Table 4. The numbers of test sera found positive and the geometric mean titers obtained in the IF and IHA tests, by classification of the sera according to the clinical form of the disease experienced by the subjects providing the sera.

		Acute (N = 10)		Intestinal (N = 14)		Hepatointestinal (N = 10)		Hepatosplenic (N = 10)		Uninfected (N = 93)	
Test	Antigen	No. pos.	GMT	No. pos.	GMT	No. pos.	GMT	No. pos.	GMT	No. pos.	GMT
IgG IF	Cercaria	10	3.3	11	2.1	10	2.5	9	2.4	4	<0.1
	Schistosomulum	10	3.1	12	2.3	10	2.7	10	2.7	0	0
	Adult worm	10	3.1	14	2.5	10	2.9	10	3.2	0	0
IgM IF	Cercaria	10	2.6	13	1.5	10	1.7	9	1.8	0	0
	Schistosomulum	10	2.5	12	1.5	7	1.6	9	1.8	0	0
	Adult worm	10	2.9	13	1.8	10	2.1	10	2.0	0	0
IgA IF	Cercaria	9	2.1	0	0	0	0	1	<0.1	0	0
	Schistosomulum	9	2.1	0	0	0	0	1	<0.1	0	0
	Adult worm	9	1.8	1	<0.1	2	<0.1	1	<0.1	0	0
IHA	Cercaria	10	2.8	11	1.4	10	2.3	8	1.9	0	0
	Schistosomulum	10	2.9	6	1.3	9	1.8	9	1.6	0	0
	Adult worm	9	1.6	14	2.1	10	2.1	10	2.4	10	0.7

Regarding IgG antibodies, the tests using cercaria antigen gave a higher GMT for acute sera than for the various classes of chronic sera (Student's t test values were all higher than 2.101, d.f. = 18, at the 0.05 level.)

With respect to the IHA tests, those using the cercaria antigen yielded results generally similar to those published elsewhere (*7, 29, 30*), despite other investigators' use of a sonicated cercaria extract as antigen. In general, cercaria antigen showed greater reactivity with acute serum antibodies than did the adult worm antigen; conversely, the adult worm antigen was more reactive than the cercaria antigen with chronic serum antibodies.

False positive results were obtained with four sera in the IgG IF test using cercaria antigen and with 10 sera in the IHA test using adult worm antigen (see Table 4). Although the specificity of both the IF and IHA tests needs to be studied further, the rates of false positive results in our previous[5] as well as in our present work are lower than those reported elsewhere using different immunoenzyme assays (*31*).

False positive results are thought to derive from cross-reactivity prompted by other unrelated helminthiases (mainly in multiple parasitic infections) (*31*), allergies to other nonpathogenic cercariae (*1, 32*), and host-like tissue components present on adult worm tegmentum (*33*). This could help to explain why, in the tests assessed here, schistosomulum antigen cultivated *in vitro*, rather than the cercaria or adult worm antigens, seemed to yield more specific results. It should also be mentioned that both cercaria and schistosomulum antigens are now being evaluated by means of immunoenzyme assays such as ELISA, ELIEDA,[6] and Dot-ELISA.

CONCLUSION

The test results described here point up the possibility of utilizing cercaria or schistosomulum antigen as an alternative to conventional adult worm antigen in serologic tests for the diagnosis of schistosomiasis mansoni. Despite the stage-specific antigens present in each of the developmental parasite stages investigated in this study, common antigenic components seem to participate predominantly in the IF and IHA tests, giving all three types of antigens similar diagnostic efficiency in all the tests performed.

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[6]ELIEDA = enzyme-linked immunoelectrodiffusion assay (a type of counterimmunoelectrophoresis associated with an immunoenzymatic assay).

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Environment and Health

The United Nations Conference on Environment and Development (3–14 June 1992) in Rio de Janeiro underlined health as a key factor in global sustainable development. Several chapters of Agenda 21, the framework for action produced by the "Earth Summit," address issues affecting health. Chapter 6, which is devoted exclusively to health, states in its introduction, "Health and development are intimately interconnected. Both insufficient development, leading to poverty, and inappropriate development, resulting in overconsumption, coupled with an expanding world population, can result in severe environmental health problems in both developing and developed nations. . . . Countries ought to develop plans for priority actions, drawing on the program areas in this chapter. . . . An appropriate international organization, such as WHO, should coordinate these activities."

This challenge provides support for a new WHO environmental health strategy, which the 45th World Health Assembly in May 1992 requested the Director-General to formulate. This strategy will be based on the report "Our Planet, Our Health" of the WHO Commission on Health and Environment.

Source: World Health Organization, Press Release WHO/42, 19 June 1992.