Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. 76(2) :103-109, abr./jun. 1981

SCHISTOSOMA MANSONI ANTIGENIC EXTRACTS OBTAINED BY DIFFERENT EXTRACTION PROCEDURES

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Solubilization of Schistosoma mansoni *antigens was obtained by agitation of adult worms in a 3M KCl solution. The protein contents of the KCl extracts varied from 0.35 to 0.96 mg/ml. Sera from 97 patients with hepatointestinal shistosomiasis and viable eggs in stools from a Brazilian endemic area were studied by immunoelectroosmophoresis and Ouchterlony immunodiffusion methods with the KCl extract and with another antigen, obtained by homogenization of adult schistosomes in saline. The rate of positiveness of immunoprecipitation detections by immunoelectroosmophoresis with the KCl extract was 53.5%. A correlation was verified between methods of detection and extraction procedures, resulting in a better association of the extract obtained by agitation in 3M KCl and immunoelectroosmophoresis.*

For the purpose of solubilizing *Schistosoma mansoni* antigens to allow their immunochemical characterization and purification, several methods are being applied. Smithers & Williamson (1961) employed the Ouchterlony agar-gel diffusion technique, demonstrating the existence of antibodies against egg and cercarial antigens in the serum of infected rhesus monkeys, using as antigen water extracts of eggs and cercariae homogenates. Biguet, Capron & Tran Van Ky (1962) reported the results of electrophoretic and immunoelectrophoretic studies on soluble antigens of *S. mansoni* using saline extracts (0.018M) of adult worms. Kent (1963) performed immunochemical analyses of larval and adult forms, also using water extracts of defatted parasites homogenized at pH 7.0 – 7.2 with EDTA and subsequently sonicated. Sadun, Schoenbechler & Bentz (1965) studied antigenic components of eggs, cercariae and adult worms, also delipidized, using extraction by homogenization in triethanolamine buffered saline for cercariae and phosphate buffered saline for egg and adult worm excretions and secretions. Other studies on antigens and immunoprecipitins in schistosomotic infection were carried out with homogenized extracts of adult worms and/or larval forms of *Schistosoma mansoni* in saline by Kagan & Norman (1963); Silva & Ferri (1965); Damian (1967), or in water by Olgivie, Smithers & Terry (1966) and extracts obtained by homogeneization in 0.85% NaCl solu-

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Received for publication on November 19th, 1976 and accepted on February 12th, 1981.

tion and ultrasonication. Hillyer & Ritchie (1967) and Kusel (1972) studied the composition of surface proteins in *Schistosoma mansoni* membrane, treating the worms with 0.5% saponin in 3% calcium chloride. Murrell, Vannier & Alimed (1974), compared by immunodiffusion a crude culture antigen (containing excretory and secretory antigens) obtained from *in vitro* culture of adult worms in a chemically defined medium with extracts obtained by freeze-thawing of adult worms and by treatment in 3M KC1. We have also verified that 3M KC1 solution is efficient in the extraction of antigens from *Schistosoma mansoni* adult worms (Scapin & Tendler, 1975a). Recently, Deelder et al (1975) described the comparison of immunoprecipitation techniques for the purpose of schistosomiasis diagnosis using antigens obtained from adult worms, eggs and snails at 14% (w/v) by homogenization in distilled water and by freeze-drying.

This paper reports the comparison of *Schistosoma mansoni* adult worm extract obtained by agitation in 3M KC1 and extract obtained by homogenization in saline, using immunoelectroosmophoresis and Ouchterlony immunodiffusion test.

MATERIAL AND METHODS

Collection of adult Schistosoma mansoni

Guinea pigs (500g) and swiss mice were infected by allowing each animal to wade for 30 min. in a jar with 30 ml (mice) and 50 ml (guinea pigs) of water containing cercariae from *Biomphalaria glabrata,* previously infected with miracidia from the laboratory of schistosomiasis – Centro de Pesquisas René Rachou – Belo Horizonte/MG. The adult worms were harvested from mice and guinea pigs after 6–7 weeks infection, by perfusion of hepatic portal system and mesenteric veins with 0.85% saline (Pellegrino & Siqueira, 1953). Worms collected by this method were stored in a small volume of saline with 1 : 5,000 merthiolate at – 10°C.

Antigens

Extraction with 3M KC1 (EKC1)

Adult worms of *Schistosoma mansoni* were suspended in a 3M KC1 solution at 13 to 15% (w/v) and submitted to agitation in an alternating mechanical agitador at 250 cpm for periods varying from 2 to 12 hrs at room temperature (20°C). Following agitation the worms were maintained overnight in solution and then removed. Following centrifugation at 10,000 for 60 min. at 4°C the supernatant was thoroughly dialyzed against 0.15M NaC1 solution at 4°C and designated EKC1. Extracts of lyophilized worms were also prepared in 3M KC1 solution at 13 to 15% w/v.

Adult worm homogenate (EHOM)

Schistosomes in saline (w/v as for the EKC1) were homogenized in a Potter Elvehjem glass homogenizer as previously described by Scapin and Tendler (1975b). Merthiolate (1 : 10,000) was added to the extracts and these were stored in a freezer.

The protein contents of EKC1 and EHOM were determined by the method of Lowry et al (1951).

Antiserum

Human sera collected from patients with schistosomiasis (hepatointestinal form) from a Brazilian endemic area of Minas Gerais were utilized. Viable eggs were found in the stools of all patients. The sera were frozen and stored for 6 – 12 months before being utilized.

A control group of 15 normal adults (18 – 25 years old), inhabitants of a non-endemic area, was also tested.

Electrophoresis

Electrophoresis of the extracts (EKC1 and EHOM) was carried out in differential gel with 8%, 6% and 4.5% layers of acrylamide gel in 1.5M Tris sulphate buffer, pH9, in a Shandon apparatus; 100 μl of each extract incorporated in sucrose was added to the superior layer (4.5%). Electrophoresis was then carried out during 2.30 hrs with constant current of 6 mA/tube. The gels were stained with 0.1% amidoschwartz solution in 7% acetic acid and distained in 7% acetic acid (Davis, 1964).

Immunodiffusion and immunoelectroosmophoresis

For detecting immunoprecipitation reactions between the antigens (EKC1 and EHOM) and the human sera, the Ouchterlony gel immunodifusion (ID) and immunoelectroosmophoresis (IEOP) methods were used.

For the ID method microscope slides were used. Diffusion was carried out for 48 – 72 hrs. The slides were covered with 2 ml of a 1% agar solution in Na barbital buffer, pH 8.4. Wells 3 mm in diameter and 4 mm apart were filled with antigen or human serum. After diffusion the slides were repeatedly washed in 0.85% NaC1 with 1 : 10,000 merthiolate, followed by water. They were then dried and stained with 0.25% Coomassie Blue in a 45% metanol and 10% acetic acid solution and destained in a 45% metanol + 10% acetic acid + 45% water solution for 20 min.

IEOP was carried out as previously described by Scapin & Tendler (1975b), with the following modifications: a discontinuous buffer system was used, being the reservoir buffer 0.05M Na barbital buffer. Glass plates were covered with 0.6% agar (Difco) in 0.13M Na barbital buffer (μ= 0.063), pH 8.4. On each plate wells 3 mm in diameter and 4 mm apart were made. Constant current of 2.8 mA/cm of gel was applied during 45 – 50 min., giving a voltage in the gel of 130v. After IEOP the plates were washed, dried, stained and destained as described above for the ID method. The KC1 extracts used for the tests with IEOP and ID methods were those which periods of extraction in 3M KC1 had varied from 6 to 12 hrs.

RESULTS

By the IEOP method using as antigen EKC1, immunoprecipitation reactions were oberved in 52 out of the 97 tested sera and with EHOM we observed positive reactions in 18 out of the total of 97. By ID method, out of sera tested with EKC1, immunoprecipitins were detected in 5, and from the 93 tested sera with EHOM, 13 presented immunoprecipitation reactions.

With the values indicated in Table I fixing the methods of IEOP and ID, the hypothesis of independence between the positiveness and the method of extraction was tested. For IEOP we rejected the independence hypothesis and a better association between KC1 – IEOP was considered ($P<0.01$). For ID we accepted the independence.

Table II shows the results of 30 sera analysed simultaneously by IEOP and ID; fixing the method of extraction (agitation in KC1) it resulted $P<0.01$ for KC1 – IEOP.

None of the normal sera tested against EKC1 by IEOP presented immunoprecipitation lines.

The extracts of fresh worms with components solubilized by 3M KC1 during 2 – 6 hrs and those obtained with lyophilized worms (period of extraction 6 hrs), showed different electrophoretic patterns (Fig. 1). Protein concentration of EKC1 and EHOM was as follows: EKC1 : 0.35 to 0.95 mg/ml and EHOM 1.5 to 2.7 mg/ml. The number of precipitin bands detected with IEOP varied from 1 – 3.

TABLE I
Correlation between IEOP and ID using EKC1 and EHOM

Extraction Procedure	*Detection Procedure*					
	IEOP			*ID*		
	positive sera	*negative sera*	*total of tests*	*positive sera*	*negative sera*	*total of tests*
EKC1	52 (35)	45 (62)	97	5 (4.7)	28 (28.3)	33
EHOM	18 (35)	79 (62)	97	13 (13.3)	80 (79.7)	93
TOTAL	70	124	194	18	108	126

$\chi^2 = 25.84$ $\chi^2 = 0.03$

(+) – Expected values in parenthesis.

TABLE II
Results of simultaneous analysis of 30 sera with EKC1 and EHOM, by IEOP and ID.

Detection Procedure	*Detection Procedure*					
	EKC1			*EHOM*		
	positive sera	*negative sera*	*total of tests*	*positive sera*	*negative sera*	*total of tests*
IEOP	15 (10)	15 (20)	30	2	28	30
ID	5 (10)	25 (20)	30	5	25	30
TOTAL	20	40	60	7	53	60

$\chi^2 = 7.50$

(+) Expected values in parenthesis.

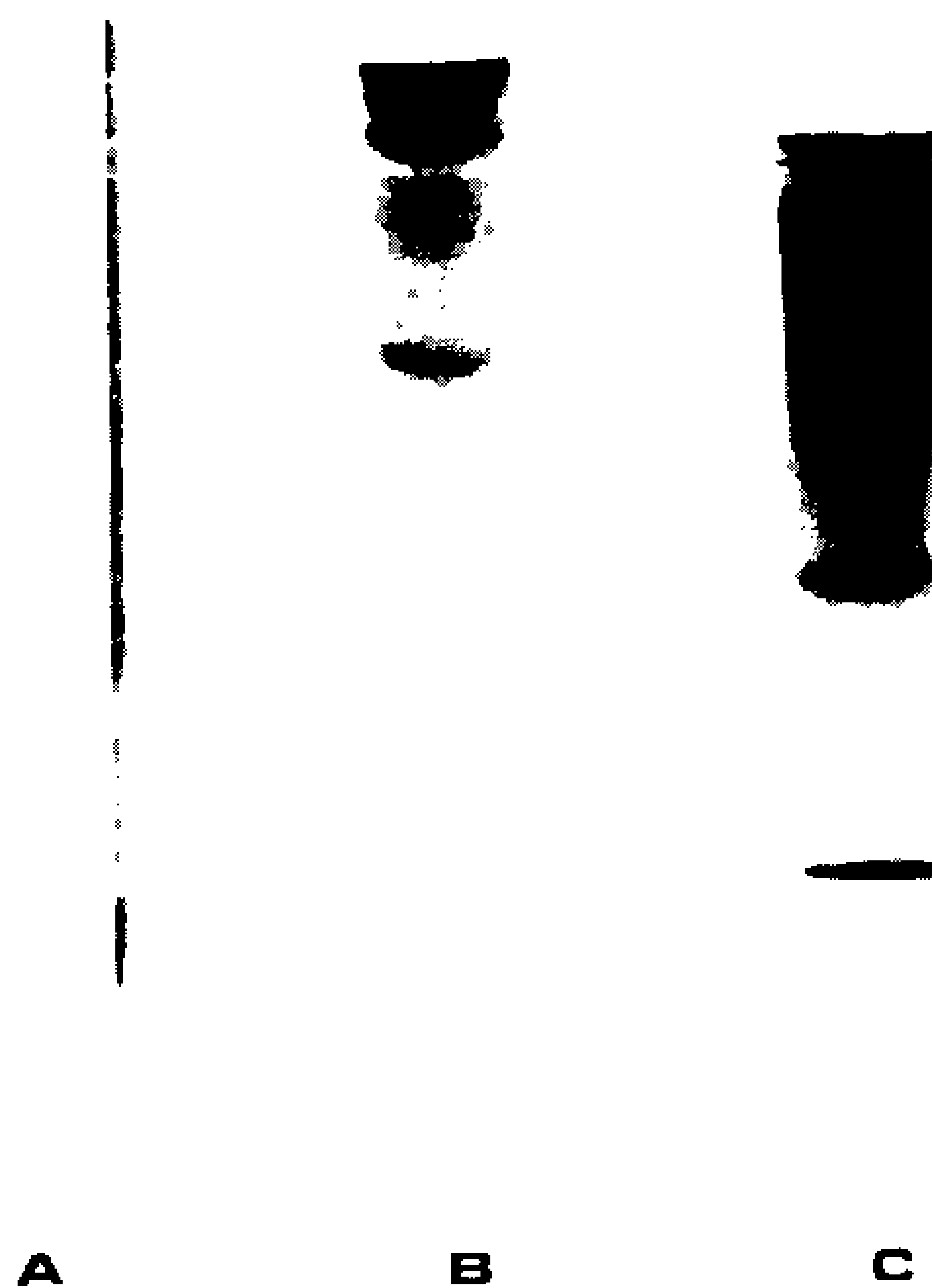

Fig. 1 – Disc gel electrophoresis of 3M KC1 *Schistosoma mansoni* extracts with agitation for 2 hrs (A) and 6 hrs (B) of fresh worms and agitation for 6 hrs of lyophilized worms (C).

DISCUSSION

IEOP was previously indicated as a method applicable for the detection of immunoprecipitation in schistosomiasis and referred to as possibly more advantageous than other methods of immunodiffusion by Scapin & Tendler (1975b). The modifications introduced here, i.e., discontinuous buffer system and staining by Coomassie Blue, increased immunoprecipitation detections, making the IEOP method more sensitive. In the present paper the results suggest a probable relationship between the IEOP method and the extraction of antigens by agitation in 3M KC1 solution. The number of reactions of immunoprecipitation detected in 97 sera tested against EKC1 by IEOP resulted in 53.5% of positiveness. This result, still not satisfactory for diagnostic purposes, is superior to the number of positive cases presently detected with the Ouchterlony immunodiffusion method with EKC1 (15.1%) and also superior to the positive cases (37.9%) reported by Silva & Ferri (1965), using the Ouchterlony test in 29 sera of hepatointestinal form against an adult worm homogenate. Other authors found different rates of positivity: Kent (1963) found 90% of positiveness in 30 cases, using immunodiffusion and water extracts of adults and cercariae.

Kagan & Norman (1963) had similar results in 17 cases; Hillyer & Ritchie (1967) had 44 positive in 63 sera from chronic cases; Deelder et al (1975) reported a positivity rate of 100% using IEOP on agarose and on cellulose acetate, but, although referring to the use of adult, egg, and snail antigens prepared by homogeneization and freeze-drying, they specified neither which antigens were tested against which sera, nor the clinical form of the patients whose sera were tested. Also, they relate false-positive reactions not found by us.

The analysis of the results suggests that may exist a difference in the antigenic content of the two extracts (EKC1 and EHOM) in respect to fractions responsible for the appearance of immunoprecipitation lines in IEOP test against human sera. 3M EKC1 could possibly release antigens which would not be present in EHOM.

Another possibility to be considered is that 3M KC1 might potentiate antigenic determinants during the extraction procedure. Our results indicate that in terms of positiveness of immunoprecipitation detected by IEOP there exists a dependence of the used method of extraction, while with ID there is no dependence. We have not yet achieved to find a satisfatory explanation for this. With the purpose of eliminating interference of 3M KC1 in IEOP, the EKC1 were exhaustively dialyzed.

Murrell, Vannier & Alimed (1974), also by immunodiffusion, comparing an antigen of adult worm excretion and secretion extract obtained by agitation of schistosomes in 3M KC1, observed antigens in common to the two extracts, referring, however, the use 3M KC1 as an attempt to obtain adult worm membrane antigens.

With electrophoresis in acrylamide gel the results of 2 hrs agitation showed a smaller quantity of extracted material than that obtained by agitation for 6hrs. Our experience shows that with extraction for more than 6hrs (6 to 12hrs) one may obtain a larger number of positive reactions by IEOP. However, a shorter time of extraction (2 hrs) does not hinder the solubilization of antigens and may be more convenient for the isolation of antigenic fractions for preparatory purposes. The pattern of acrylamide gel electrophoresis of the extract of worms lyophilized prior to the extraction with 3M KC1 was poorly defined, which may be explained as a possible denaturation. Houssain, Vannier & Murrell (1975) reported that the lyophilization of adult worms prior to extraction by ether reduces the activity and may denature alergens. Due to these observations we only took into consideration in the present work results with EKC1 prepared from fresh worms with agitation for 6 to 12 hrs.

Further studies to characterize the antigens extracted by 3M KC1 are needful but in the here described results there is evidence of the effective capacity of extraction of 3M KC1 with regard to *Schistosoma mansoni* antigens as well as of the correlation between the antigens present in EKC1 and immunoprecipitins in the sera of schistosomotic patients, as revealed mostly with the IEOP test.

RESUMO

Foi obtida a solubilização de antígenos do *Schistosoma mansoni* por agitação de vermes adultos em solução de KC1 3M. O teor protéico dos extratos de KC1 variou de 0,35 a 0,96mg/ml. Foram testados pelos métodos de imunoeletroosmoforese (IEOP) e dupla imunodifusão (Ouchterlony), 97 soros de doentes de área endêmica brasileira de esquistossomose, forma clínica hepatointestinal e com exames coprológicos positivos para *S. mansoni*, com o extrato de KC1 e outro antígeno obtido pela homogenização de vermes adultos em salina. A taxa de positividade das reações de imunoprecipitação por IEOP com o antígeno extraído pela ação do KC1 3M foi 53,5%. Foi verificada a correlação entre os métodos de detecção e de extração resultando numa melhor associação entre o extrato obtido por agitação no KC1 3M e a IEOP.

ACKNOWLEDGEMENTS

We are especially thankful to Dr. José Pellegrino* for supplying the lyophilized worms and to Dr. Naftale Katz for supplying the human sera and infected snails. We wish to acknowledge Mr. Plínio Chrispim for technical assistance and statistical analysis.

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*Died in 1978.

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