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Purification of the Clonorchis Antigen using a Diethylaminoethyl-Sephadex A50 Column*

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ABSTRACT

Antigens for the diagnosis of clonorchiasis have been isolated from the adult *Clonorchis sinensis*. Following extracttion with a phosphate-citrate buffer solution, ultrasonic disintegration, and defatting with a chloroform-methanol mixture, the antigens were fractionated in a Diethylaminoethyl-Sephadex (DEAE-Sephadex) A 50 column using sodium chloride eluates. The antigens were evaluated by carrying out both skin tests and stool examinations to determine their sensitivity, specificity and cross reaction. These DEAE fractionated antigens show less cross reaction than Melcher's antigen. DEAE fractions were protein(280 mμ peak) and nucleoprotein (260 mμ peak) as indicated by spectrophotometry.

These purified fractions represented by the 260 mμ and 280 mμ peaks were not free of cross reactions nor were they completely specific for clonorchiasis. It is therefore suggested that further attempts be made to eliminate cross reactions and to enhance specificity by a more detailed and complete method of purification or of preparation of the chemical components.

INTRODUCTION

Since Chung et al. (1955) carried out skin tests on patients with *Clonorchis sinensis*, several workers (Hunter, 1958, Sadun, 1959a)have purified the clonorchis antigens for the immunodiagnosis of clonorchis. This purification is necessary, since crude antigens contain factors which produce extensive, non-specific reactions, and cross reactions remained. Sadum(1959a) and Hunter(1958) have reported a cross reaction to paragonimiasis of 57.0 and 44.7 per cent respectively.

While the apparent solution of this problem lies in the purification of the antigenic components, relatively few investigations for *Clonorchis sinensis* have been conducted along this line.

The authors isolated purified antigens from adult *Clonorchis sinensis* using Diethylaminoethyl-Sephadex (DEAE-Sephadex) anion exchange columns, after preliminary extraction by phosphate-citrate buffer solution after defatting with a chloroform-methanol mixture. The antigens isolated by the DEAE-Sephadex column showed a low degree of cross reactions but retained their antigenicity.

MATERIALS AND METHODS

Worm collection: Antigens for the intradermal tests were prepared from adult *Clonorchis sinensis* obtained from cats, dogs and rabbits experimenta lly infected four months previously with *C. sinensis* metacercariae. Metacercariae were obtained by the pepsin digestion of fish, or raw fish(Hemiculter kneri) collected in an endemic area in Liu-ying (Taiwan)(Kuntz, 1960a), and were fed to the experimental animals. The animals were sacrificed four months later and intact worms removed from the biliary passages. The worms were washed repeatedly with physiological saline, and rinsed with distilled water. They were transferred to ampules, rapidly frozen, lyophilized, and stored in vacuum

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sealed-vials at-50°C.

Antigen preparation:

Preliminary extraction: Worms were collected individually from stored vials using forceps so that no surrounding worm powder would adhere. A 100 mg. quantity of lyophilized adult worms was suspended in 25ml. of 0.1 M phosphate buffer(pH 7.0) containing 0.015 M sodium citrate. The suspension was transferred to a tissue grinder and ground thoroughly in a $CaCl_2$-ice bath for ten minutes. This suspension was disintegrated in an ultrasonic disintegrator at 20 KC/Sec, 8-9 amp for 7 minutes and then homogenized again in a $CaCl_2$-ice bath for 5 minutes. The suspension was transferred to a large tube and gently shaken for 10 minutes with 20 ml. of chloroform-methanol(4:1). It was then placed in a refrigerator at 4°C for one day of constant stirring. The suspension was homogenized manually for 5 minutes and centrifuged at 1000g for 30 minutes at 4°C, thus separating the contents of the tube into three layers. The aqueous layer was collected with capillary pipette for antigen preparation.

Purification processes: DEAE-Sephadex A 50 (medium)* 2.5 gm. was equilibrated with 0.01 M phosphate buffer(pH 7.0). The column(1.2cm internal diameter, 30 cm in length) was gradually filled with suspension of DEAE-Sephadex A 50. The phosphate-citrate- buffer extract of worms was slowly transferred to the DEAE column. The column was eluated with 0.5 M NaCl solution in 0.01M phosphate buffer(pH 7.0). The 280 mμ peak wave as indicated by a Beckman Recording Spectr ophotometer was collected. Another fraction was eluated with 1.0 M NaCl solution in 0.01 M phosph ate buffer (pH 7.0). The fraction indicated by 260 mμ peak was collected. These two fractions were dialyzed on a Sephadex G 25 column equilibrated with 0.01 M citrate buffer(pH 7.0). The components were recovered by eluation with the same buffer. The flow rate did not exceed 0.5 ml per minute. Fractions were lyophilized and stored at-20°C in a bottle with rubber stopper.

The lyophilized extract was diluted with 1:10,000 merthiolate solution, and the purified fractions used for skin test antigens.

Intradermal tests: Three towns in Taiwan were chosen for field testing of the antigens. Clonorchiasis is endemic in Meinung(Kaohsung)(Kuntz, 1961a) while paragonimiasis is known to be endemic in Ali-lau and Shin-Keng(Taipei)(Kuntz, 1961b).

A total of 252 adult volunteers were selected for the clonorchis study. Skin tests for checking cross reactions were performed on 51 people previously proven to be positive for paragonimiasis. The diagnosis in these cases had been made by Kuntz (1961b) on the basis of stool and sputum examinations. The age range was from 2 to 63 years.

Intradermal tests were performed with 0.02 ml. of antigen using a 27 gauge needle and a tuberculin syringe. Injections were given into the volar surface of the forearm. A small wheal about 20mm² in area was raised each time. DEAE-Sephadex fraction 1, 11 and Melcher's antigen were injected into the right forearm, and paragonimus antigen* and control buffer containing 0.01 M citrate-saline plus merthiolate (1:0.000) were injected into the second forearm. The concentration of antigens used was 0.05mg of protein per ml of DEAE fraction 1 and 0.066 optical density for DEAE fraction 11, and 0.1 mg of protein per ml of Melcher's antigen. The areas of ensuing wheals were estimated after 15 minutes by means of a transparent sheet on which calibrated rings outlined the areas between 20 mm² and 700 mm². When the transparent film was reviewed, positive, dubious, and negative standards were considered to be over 60mm², 50-59 mm² and less than 49 mm² respectively according to a modification of Kagan's method(1961).

Stool examination: Stool specimens from the persons tested were studied by the merthiolate-Iodine-Formalin concentration method (Blagg, et al. 1955).

Complement-fixing tests: A complement-fixing test of antigens was carried out using Kolmer's method (1951). Antisera of clonorchiasis and paragonimiasis were obtained from the experimental animals (dogs and cats) which had been infected four months previously with *C. sinensis or P. westermani* metacercariae. Serum was taken from the

* *DEAE-Sephadex A 50(medium) obtained from PHARMACIA, Uppsala sweden*

* *Paragonimus antigen was supplied from Mr. Liu working at US Naval Medical Research Institute 2*

animals with stools positive for the clonorchis or paragonimus ova.

RESULTS AND DISCUSSION

The fractions of DEAE column employed were shown by spectrophotometry to contain protein and nucleoprotein respectively. However, many authors have reported that the chemical components which possess antigenicity in parasites were polysaccharide (Cmeric, 1952), glycoprotein(Kent, 1960), lipoprotein (Korach, 1961), polypeptide (Maurer, 1959), and protein(Sadun, 1959a).

The preparation of clonorchis antigen was based upon the following background works. Prior to the DEAE anion exchange column analysis, the authors tried to eliminate the lipid fraction with a chloroform-methanol mixture. The lipid fraction is responsible for cross reaction(Sadun, 1959 b Chaffee, 1945 Taliferror, 1931). Many organic solvents have been used by various investigators to eliminate the lipid fractions: ether(Taliaferro, 1931), anhydrous ethanol (Taliaferro, 1931), petroleum ether (Melcher, 1943) and cold anhydrous ether (Sadun, 1959 b). However, cross reactions remained in a high percentage of cases(Sadun, 1959b). Recently, Sleeman (1960) has reported that the chloroform-methanol mixture eliminated a non-specific substance and reduced cross reactions in schistosoma antigen studies. Sevag(1938) has reported that chloroform did not denature or injure the protein components in nucleoprotein preparation. The authors used a pho-

Table 1. Intradermal test for clonorchis sinensis with different preparations of antigen from adult worms

Name of antigens	Number of people examined	Positive over60mm²		Dubious 150-59mm²		Negative below49mm²	
		No.	%	No.	%	No.	%
DEAE fraction 1	252	76	30.15	15	5.15	161	63.8
DEAE fraction 11	252	75	29.75	19	7.57	158	62.69
Melcher's antigen	241	77	31.75	21	8.71	143	59.33

Table 2. Wheal size produced by antigens according to age and sex

Age groups in years	Antigen*	Number of people exam			Wheal size mm²(mean)		
		Male	Female	Both sex	Male	Female	Both sex
16~29	1	12	2	14	50~170	60~120	50~170(80)
	11	16	3	19	50~150	60~100	50~150(73.8)
	111	18	5	23	50~130	50~ 80	50~130(72)
30~39	1	25	8	33	50~120	50~120	50~120(73)
	11	25	6	31	50~150	50~ 90	50~150(73)
	111	26	7	33	50~160	50~ 90	50~160(73)
40~49	1	18	3	21	50~170	60~100	50~170(81.4)
	11	21	3	24	50~170	50~ 80	50~160(82.1)
	111	19	2	21	60~170	50~ 90	50~170(80.0)
50~59	1	10	3	13	50~130	50~100	50~150(89.2)
	11	11	2	13	50~160	50~ 80	50~150(85.0)
	111	9	2	11	60~160	60~ 80	60~160(97.2)
60~69	1	5	3	8	50~130	60~150	50~150(89.0)
	11	3	2	5	50~150	60~180	60~160(88.0)
	111	6	2	8	60~150	50~100	60~150(82.5)
70~79	1	1	1	2	100	100	100 (100)
	11	1	1	2	100	100	100 (100)
	111	1	1	2	120	100	100~120(110)

Antigen*1: DEAE fraction 1 11: DEAE fraction 11 111: Melcher's antigen

sphate buffer solution for extraction. Others have used saline(Chung, 1955), acid or alkaline(Sadun, 1959a), Coca's solution(Davies, 1954), phenol(Wright, 1947) and alcohol(Hunter, 1958). A phosphate buffer has been used widely for enzyme or protein purification without cauing denaturation(Stephen, 1960 Derrien, 1952. Morton, 1955). Several authors have reported that the nucleoprotein possesses antigenicity(Sevag, 1938, Grigor (Yan, 1960). The genetic material in all living systems and in all cellular forms appeared to be a nucleic acid, deoxyribonucleic acid(DNA). In higher forms DNA is associated closely with protein(George, 1957). A citrate buffer(Kay, 1952, Stern, 1951) solution and an ultrasonic disintegrator(Diena, 1962) have been used for the destruction of nucleoproteinase. Several investigators have reported the use of DEAE anion exchange column for protein purification (Sober, 1956, Bieserte, 1961) or for antigen preparation(Palmstierna, 1960).

Table 3. Wheal size due to various concentrations of antigens (in mm²)

1. DEAE fraction 1 (concentration expressed by) protein at 280mμ)

Dilution	1 time (0.7mg/ml)	6	10	12	14	16
Case 1	60mm²	60	60	60	50	50
Case 11	100	120	120	100	80	100

2. DEAE fraction 11 (concentration expressed by optical density at 260mμ)

Dilution	1 time (0.396 O.D)	3	6	12	20
Case 1	60mm²	60	60	50	40
Case 11	100	100	120	100	80

Table 4. Wheal size due to various volumes of antigen

Volume / Injection	0.02 ml	0.04	0.06
Control	20 mm²	40	50
Antigen	80	100	120

Table 5. Cross reaction to proven paragonimiasis cases

Kind of antigen	Total (%)	Positive (%)	Dubious (%)	Negative (%)
DEAE fraction 1	51(100)	6(11.8)	5(9.8)	40(78.4)
DEAE fraction 11	51(100)	0(0.0)	3(5.9)	48(94.1)
Melcher' santigen	51(100)	10(19.6)	7(13.7)	33(66.7)

Table 6. Percentage of cross reaction with various antigens to proven paragonimiasis

Antigen	Number of people examined	Cross reaction %	Reporter
Saline extract	18	100	Chung et al.(1955)
Acid soluble (Melcher's)	93	57	Sadun et al.(1959b)
Water soluble	47	44.7	Hunter et al.(1958)
DEAE 11 fraction 1	51	21.6	J.H. Kim(1963)
	51	5.9	//
Melcher's	51	33.3	//

Table 7. Relation of intradermal test and stool examination

Intradermal test Reaction	Antigen*	Stool examination: Positive No.	Positive %	Negative No.	Negative %	Total No.	Total %
Positive	1	55	73.33	20	26.6	75	100
	11	51	70.83	21	29.16	72	100
	111	51	69.91	23	31.08	74	100
Dubious	1	12	70.58	5	29.41	17	100
	11	12	60.0	8	40.0	20	100
	111	9	42.85	12	57.14	21	100
Negative	1	23	14.46	136	85.53	159	100
	11	27	16.98	132	83.01	159	100
	111	26	17.68	121	82.31	147	100

Antigen*: 1: DEAE fraction 1 11: DEAE fraction 11 111: Melcher's antigen

Evaluation of the various antigen preparations was made by intradermal tests, complement-fixing tests and stool examination. Table 1 shows the intradermal reaction rates for various clonorchis antigens. The incidence of positive reactions was not significantly different in these cases. Table 2 shows the wheal size of the skin reactions to the various antigens. Three different antigens failed to show significant differences in wheal size. The wheal sizes produced by all three antigens were smaller than those reported by Sadun(1959b)(136mm²) and by Kuntz(1961b)(113mm²). In the control injection, the wheal size averaged 23 mm². This is less than the 37mm² and 40mm² reported by these authors (Kuntz, 1961b, Sadun, 1959b). This result may not consider to be a function of antigen concen tration but rather to be due to the volume of antigen used for skin testing. See Table 3 and 4. However, the degree of dermal reaction in positive cases has no correlation with severity of the infection (Taliaferro, 1931).

Three antigens were checked for cross reaction in 51 cases of paragonimiasis westermani. The res ults are shown in Table 5. The percentage of cross reactions with various antigens along with other authors' data, are summarized in Table 6. The DEAE fractions showed a lower degree of cross reactions to paragonimiasis than was found with Melcher's antigen. The DEAE fraction 11 showed the lowest cross reaction.

Table 7 shows the relation of the intradermal test to the stool examination. The two were closely correlated. Among those patients whose stool were positive for clonorchis ova, negative intradermal reactions were found in 14.46, 16.9 and 17.68 per cent with the DEAE fraction 1, 11 and Melcher's antigen respectively. This data showed that the DEAE fractions gave less false negative results than Melcher's antigen. Sadun(1959b) has reported 19 percent false negative reactions in skin testing

Table 8. Complement-fixing test of various antigens

1. Complement-fixing tests in high concentraion(0.7mg of protein per ml of antigen 1, 0.70 optical density at 260 mμ of antigen 11)

Serum	Antigen*	Initial serum dilution				
		8	16	32	64	128
Clonorchis	1	4	4	4	4	3
	11	4	4	4	4	4
	111	Did not check				
Paragonimus	1	4	4	4	3	2
	11	4	4	4	4	4
	111	Did not check				

Antigen control	with comp.	0
	without comp.	4
R. B. C. control		4
Hemolysin control		0
Comp. Units	1:37	
Ambo.	1:2500	
Comp. titer Antigen		1.35……0
Serum control Clonorchis		11.45……0
Serum control Paragonimus		111.25……0

2. Complemeut-fixiug tests in low concentration (0.05mg/of protein per ml of Antigen 1, 0.066 optical density at 260 mμ of antigen 11, 0.1 mg of protein per ml of antigen 111)

Serum	Antigen*	Initial serum dilution				
		8	16	32	64	128
Clonorchis	1	0	0	0	0	0
	11	0	0	0	0	0
	111	0	0	0	0	0
Paragonimus	1	4	3	T	0	0
	11	T	0	0	0	0
	111	0	0	0	0	0

Antigen control	with comp.	0
	without comp.	4
R. B. C. control		4
Hemolysin control		0
Comp. Units	1:37	
Ambo.	1:2500	
Comp. titer Antigen		1.35……0
		11.45……0
		111.25……0
Serum control Clonorchis		0
Serum control Paragonimus		0

Antigen*: 1: DEAE fraction 1 11: DEAE fraction 11 111: Melcher's antigen Comp.: Complement Ambo.: Amboceptor

Table 9. Incidence of chlonorchiasis in Meinung town

Author	Reported date years	Examined number	Positive cases		Type of population	Method of checking
			No.	%		
Hsieh	1959	514	115	22.35	General	Stool exam.
Chow	1960	573	141	24.6	//	Intradermal
Chow	1960	133	69	51.88	//	Stool exam.
Kuntz	1961	337		34.00	//	Stool exam.
J.H.Kim	1963	252	* 1: 76 11: 75 111: 77	30.15 29.75 31.95	// // //	Intradermal test
J.H.Kim	1963	251	89	35.4	//	Stool exam.

* 1:DEAE fraction 1 antigen 11:DEAE fraction 11 111:Melcher's Antigen

Table 10. Intradermal test for clonorchiasis by sex

Sex	Antigen*	Total No.7 examin.	Positive		Dubious		Negative	
			No.	%	No.	%	No.	%
Male	1	132	59	44.69	11	8.33	62	49.96
	11	132	61	46.21	16	12.1	55	41.66
	111	125	65	52.00	14	11.2	46	36.8
Female	1	120	17	14.16	4	3.33	99	82.5
	11	120	14	11.7	3	2.5	103	85.83
	111	116	12	10.34	7	6.03	97	83.62

Antigen*: 1: DEAE fraction 1 11:DEAE Tractionte 11 111: Melcher's antigen

Table 11. Intradermal test of Clonorchis sinensis for different age groups

Age group in years	Antigen *	Number examined	Positive		Dubious		Negative	
			No.	%	No.	%	No.	%
16~29	1	74	12	16.2	2	2.7	60	81.08
	11	74	18	24.3	1	1.35	55	74.3
	111	73	19	26.02	4	5.4	50	68.5
30~39	1	78	25	32.05	8	10.25	45	57.69
	11	78	20	25.6	11	14.10	47	60.25
	111	73	23	31.5	10	13.6	41	56.16
40~49	1	58	19	32.75	2	3.44	37	63.79
	11	58	20	34.4	4	6.89	34	58.62
	111	56	17	30.35	4	7.14	35	62.5
50~59	1	24	11	45.83	2	8.33	11	45.83
	11	24	10	41.6	3	12.5	11	45.83
	111	21	11	52.38	0	0	10	47.6
60~69	1	13	7	53.84	1	7.69	5	38.46
	11	13	5	38.46	0	0	8	61.53
	111	13	5	38.46	3	23.07	5	38.46
70~79	1	5	2	40.0	0	0	3	60.0
	11	5	2	40.0	0	0	3	60.0
	111	5	2	40 0	0	0	3	60.0

Antigen *: 1: DEAE fraction 1 11: DEAE fraction 11 111: Melcher's antigen

Melche's antigen.

Prior to the intradermal tests, complement-fixing tests were done with the antigens to evaluate antigenicity and to detect any possible cross reactions in related trematode infections such as paragonimiasis westermani. The complement-fixing tests performed was of no value in checking cross reactions of antigens in the laboratory. Table 8 shows the results of the complement-fixing test with various amount of three antigens. All three antigens, at high concentrations showed antigenicity and cross reactions. In low concentration, on the other hand, antigenicity and cross reactions were not noticeable. Antisera of people with ascariasis and

Table 12. Wheal size of control injection by sex ratio

Sex	Number examined	Wheal size in mm²											
		less 20		20		30		40		50		60	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Male	132	43	32.57	49	37.12	15	11.36	22	16.6	3	2.27	0	0
Female	120	69	57.5	46	38.33	1	0.83	3	2.5	0	0	1	0.83

healthy persons did not show fixation with the three antigens.

Field studies: Table 9 shows the incidence of *Clonorchis sinensis* in Meinung along with the data available from other authors. These rates are hig her than the 24.6 per cent reported by Chow(1960). Table 10 shows the incidence of clonorchis by sex difference. Males had a higher per centage of positive dermal reactions to each antigen than did females. The percentage of dermal reactions to each antigen increased with age up to the age of 50 to 59, and tended to decrease in older individuals. The rates of clonorchis infection for different age groups are summarized in Table 11. This results showed similar findings to those reported by Chow (1960) and others(Sadun, 1959b Walton, 1959).

Table 12 shows the wheal size of the control injection by sex. The size of the wheal in males was bigger than that of the females in the control injections. In the control injections 95.8 per cent of all female and 69.6 per cent of all male had wheal less than 20mm.²

In one case a wheal of 60mm² at the site of control was identical to that of a positive reaction to clonorchis or paragonimus antigen with no positive ova in his stool. This may have an allergic reaction to merthiolate. Some of the people tested have complained of an itching sensation at the site of the injection. However, no correlation was found between itching and positivity except in two cases with 40mm² wheals and positive ova in their stools.

96 per cent of the subjects tested admitted eating raw fish and 100 per cent of those who reacted positively to clonorchis antigen and showed positive ova had eaten raw fish.

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