ANTIBODIES IN SERUM OF PATIENTS WITH CLONORCHIASIS BEFORE AND AFTER TREATMENT

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Abstract. Sera of 31 patients infected with *Clonorchis sinensis* were examined using fraction 1 antigen by ELISA during a one-year observation. The results of ELISA with Igs, IgG and IgA demonstrated high sensitivity (100%, 100% and 90%) and specificity (100%, 100% and 87%). Sera specific Igs and IgG were significantly decreased in the 3rd month after treatment with praziquantel (25mg/kg body weight in one dose), and IgA was significantly decreased in the 1st month (paired t-test, $p < 0.05$). No eggs were found in the stool after treatment. Detection of sera specific Igs, IgG and IgA by ELISA was combined with stool examination to evaluate the effect of praziquantel and the completeness of the cure.

INTRODUCTION

Clonorchis sinensis, the cause of chronic hepatic infection in the human host, is an important zoonotic disease in the Far East. This parasite also constitutes one of the major health problems in Taiwan. The fluke is a parasite infecting the fish-eating population in southern Taiwan. The prevalence rate in the endemic regions has been found to be around 50% (Chow, 1960; Clarke *et al,* 1971; Chen *et al,* 1979; Ong and Lu, 1979; Wang *et al,* 1980).

Praziquantel, an anthelmintic which has been introduced to treat clonorchiasis, has proven to be highly effective while having few side effects (Rim *et al,* 1981). The efficacy of praziquantel was evaluated by stool examination for three consecutive days. Stool examination, the most direct and economic method for detecting *C. sinensis,* is of limited value for mass screening due to poor patient compliance and diagnostic slowness. On the other hand, immunodiagnosis has many advantages including high sensitivity and specificity. A highly sensitive and specific test for antibodies to the parasite would provide a useful tool for epidemiological surveys, giving information on previous exposure to the parasite and, perhaps, some indication of prospective pathological complications. Many immunodiagnostic techniques have used crude antigen in the diagnosis of clonorchiasis (Chen and Yen, 1985; Kim *et al,* 1987). We fractionated a crude *C. sinensis* extract by gel filtration to prepare a partially purified antigen in a previous study and it proved to have good sensitivity and suitability for employment in an enzyme-linked immunosorbent assay (ELISA) system (Lin *et al,* 1990).

An individual's exposure to *Clonorchis sinensis* elicits antibody responses. Sera total or specific IgG and/or IgE increased significantly in clonorchiasis patients (Chen and Yen, 1984; Chen and Yen, 1985; Kim *et al,* 1987). Previous studies showed that sera specific IgG will change significantly before and after treatment (Chen *et al,* 1987 a,b; Kim *et al,* 1987). However, the change in other antibody classes and the role of humoral responses in *Clonorchis sinensis* infection are not yet completely understood. The change in sera antibodies is helpful to immunodiagnosis in indicating appropriate treatment and will be a good indicator for mass epidemiologic screening. Our previous study reported that sera antibody isotypes, including specific mixture of immunoglobulins (Igs; containing IgG, IgM and IgA), IgG, IgM, IgA, IgE and total IgE, increased significantly in ELISA, and specific Igs, IgG, IgA and total IgE correlated significantly to the infection intensities expressed as eggs per gram feces (EPG) (Lin *et al,* 1990). Based on these findings, the present study has conducted a monthly follow-up examination on patients with *Clonorchis sinensis* using partially purified antigen by ELISA for a period of one year to observe the change in sera antibody isotypes and the effect of praziquantel treatment.

MATERIALS AND METHODS

Subjects and test samples

Thirty-one sera were obtained from patients admitted to the special parasitic disease clinic, Chung-ho Memorial Hospital, Kaohsiung Medical College, Kaohsiung, Taiwan. The patients were infected with *C. sinensis* confirmed by stool examination for three consecutive days. The patients were between 39 and 60 years of age, 24 were males and 7 were females. All patients were treated with praziquantel, 25 mg/kg body weight in one dose, followed up by stool examination and enzyme-linked immunosorbent assay (ELISA) for one year. Stool examination was performed on three consecutive days after treatment, in the 1st and 5th week, and the 2nd, 6th and 12th months to observe any possibility of any parasitic infection during the follow-up period. However, sera were collected and examined by ELISA every month. The sera were stored at -70°C with 0.02% sodium azide until use. The sera of 30 people from a non-endemic area were used as control in the present study.

Parasites

The fresh water fish *Hemiculter kneri,* the second intermediate host of *C. sinensis,* was digested by artificial gastric juice (pepsin : HCl; 0.5%:0.8% (w/v)) for collection of metacercaria to infect guinea pigs. Adult worms of *C. sinensis* were obtained from the animal two months after infection, lyophilized and stored at -20°C until use.

Antigens

A crude phosphate-buffered extract of adult *C. sinensis* from the guinea pigs was prepared as previously described (Lin *et al,* 1990). This crude antigen was passed through a Sephadex G-200 (Phamacia Co) gel filtration column. The column was eluted with 0.15M phosphate buffered saline (PBS), pH 7.2, at a flow rate of 8 ml/hour at 4°C. The eluent was monitered at UV 254nm and collected for further analysis by ELISA. Protein concentrations were measured using a Bio Rad protein assay kit and bovine serum albumin as a standard.

Stool examination

Eggs from infected patients were concentrated by the antiformin-ether concentration method from stool specimens and counted per gram of feces (Lin *et al,* 1990).

ELISA

The ELISA was performed as previously described (Lin *et al,* 1990). Optimal dilution of antigen, serum and conjugate were determined by checkerboard titration using positive and negative reference sera. The antigen, serum and conjugate dilution, and substrate reaction time which gave the greatest difference between the optical density (OD) values for negative and positive reference sera were used in the survey. Polyethylene microtiter plates (Falcon 3912) were coated with 5 μg/ml of antigen in 0.1M carbonate buffered saline, pH 9.6 at 4°C overnight. Each well was then washed 3 times with PBS containing 0.05% Tween 20 and blocked with 1% bovine serum albumin at 37°C for 20 minutes. The plates were then washed as above, and 0.1 ml sera were added. Serum blanks, antigen blanks and positive and negative control sera were run with each plate. After incubation for 1 hour at 37°C, the plates were washed, and the conjugates of horse radish peroxidase conjugated with goat anti-human IgG, IgM, IgA, IgE (Sigma) or Igs (Cappel) were added and incubated at 37°C for 1 hour. After the addition of 1,2-phenylenediamine enzyme substrate solution (Dakopattus) as a substrate, the OD values were determined by a Titertec Multiskan Colorimeter (Flow, Finland) at 490 nm.

RESULTS

ELISA

Five fractions were isolated from the crude antigen and passed through a Sephadex G-200 column. These fractions and the crude antigen were examined by ELISA to detect antigenicity with infected sera. The crude antigen and fraction 1 antigen showed the greatest difference in OD between negative controls and positive sera. The fraction 1 antigen was revealed as having antigenicity as good as crude antigen in this study.

The ELISA test was carried out using fraction 1 antigen to detect specific antibody isotypes of clonorchiasis. Thirty-one sera from infected patients before treatment and thirty from controls were examined by ELISA. The antibody levels were expressed as OD values. The distribution of specific Igs, IgG, IgM, IgA, and IgE are shown in Fig 1 and 2. Sera specific Igs and IgG demonstrated the greatest difference in OD values between infected and control references. Taking cut-off values of Igs and IgG of 0.45 and 0.5 at 490 nm, there were no false negative or false positive results. The sensitivity

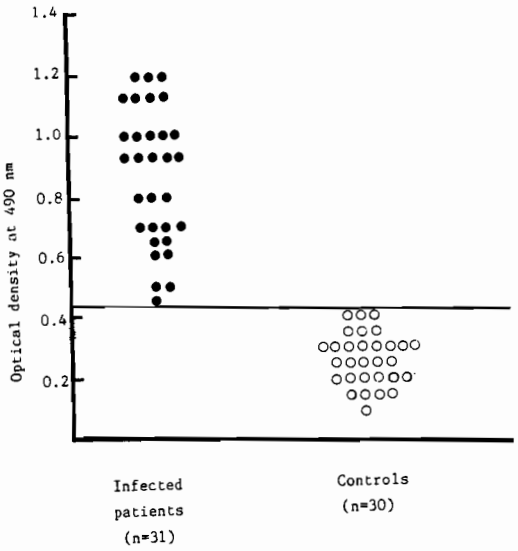

Fig 1–Seroactivity of specific Igs antibodies containing IgG, IgM and IgA against fraction 1 antigen in patients infected with *C. sinensis* before treatment. The horizontal line represents cut off values.

and specificity of antibodies by ELISA are shown in Table 1. The sera of specific Igs, IgG and IgA indicated high sensitivity and specificity.

Follow up

Thirty-one sera of clonorchiasis were followed up by ELISA to detect the pattern of antibody isotypes and the effect of praziquantel treatment. All of the patients were treated with praziquantel, 25 mg/kg body weight in one dose, and traced by stool examination to make sure no eggs were in the stool during the follow-up period. The average of EPG was 1,503 before treatment, and the results shown in Table 2 indicated no eggs in the stool in the

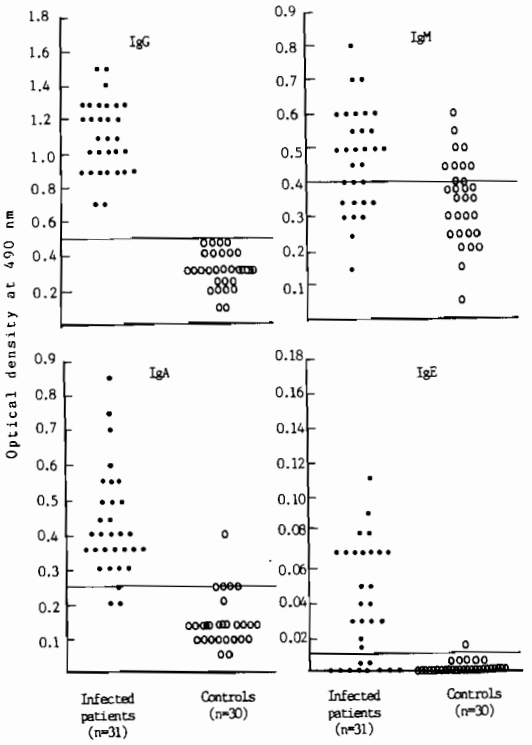

Fig 2–Seroactivity of specific IgG, IgM, IgA and IgE antibodies against fraction 1 antigen in patients infected with *C. sinensis* before treatment. The horizontal line represents cut off values.

2nd, 6th and 12th month after treatment with praziquantel. The cure rate and egg reduction rate (ERR) were both 100%. As shown in Table 3, the specific antibodies, namely, Igs and IgG decreased significantly in the 3rd month and IgA, in the 1st month after treatment (paired *t*-test, $p < 0.05$). On the other hand, specific IgM and IgE showed no significant difference before and after treatment during the one-year observation.

DISCUSSION

Immunodiagnosis of human clonorchiasis is very important for development and introduction of more reliable immunodiagnostic procedures which may facilitate detection of adult worm antigen in patients. The adult worms induce a wide range of immunological responses in both human and experimental animal hosts (Yen and Chen, 1983; Chen and Yen, 1984; Chen and Yen, 1985 a,b). This

Table 1

Sensitivity and specificity in detection of 31 patients with clonorchiasis and 30 control sera by ELISA using partial purified adult worms antigen.

Antibodies	Sensitivity (%)	Specificity (%)
Igs	100	100
IgG	100	100
IgM	61	90
IgA	90	87
IgE	68	96

*Igs containing IgG, IgM and IgA

also could be an indication for appropriate administration of the most efficient drug treatment. Such an immunological test for clonorchiasis should have a high sensitivity and extremely high specificity to avoid false positive and negative results. We believe that detection and purification of *C. sinensis* antigen may improve immunodiagnosis of human clonorchiasis. In the present study, gel filtrative separation of crude extract showed 5-fraction eluent and detected antigenic specificity by ELISA. Crude antigen and fraction 1 had similar antigenicity because the greatest difference in OD values was between positive and negative controls. Using this fraction 1 to detect 31 infected sera in specific Igs and IgG by ELISA, none was negative if the cut off was 0.45 and 0.5, and the specificity of the test

Table 2

Eficacy of praziquantel to the patients infected with *C. sinensis.*

3 × 25 mg/kg for one day	No.	EPG*	Cure (%)	EER** (%)
Before treatment	31	1,503	-	-
After treatment (2)	31	0	100	100
(6)	31	0	100	100
(12)	31	0	100	100

* Eggs per g feces, mean number in examination for 3 consecutive days
** Egg reduction rate
( ) Months after treatment for stool examination

Table 3

Serum antibody levels in patients with clonorchiasis by ELISA before and after treatment with praziquantel.

Antibodies	Before treatment	Examination after treatment	
		Significant reduction (month)**	The 12th month
Igs***	0.83 ± 0.20	0.76 ± 0.20 (3)	0.67 ± 0.20
IgG	1.10 ± 0.21	1.01 ± 0.26 (3)	0.92 ± 0.24
IgM	0.46 ± 0.16	-	0.46 ± 0.19
IgA	0.45 ± 0.18	0.40 ± 0.17 (1)	0.12 ± 0.06
IgE	0.04 ± 0.03	-	0.03 ± 0.03

* OD values at 490nm, Mean ± SD.
** Significant difference in the OD values at the beginning of reduction month intervals after treatment to those before treatment (Paired *t*-test, $p < 0.05$).
*** Igs containing IgG, IgM and IgA.

was 100%. The purposes of immunodiagnosis techniques are to prove patients are truely infected (sensitivity) and to exclude the completely non-infected subjects (specificity) (Glickman *et al,* 1987). The ELISA test for specific Igs, IgG and IgA demonstrated high sensitivity and specificity. However, recent reports have shown that crude antigen could be cross reacted with sera infected with other helminths (Chen *et al,* 1987), and the fraction 1 also exhibited cross reactivity with other helminths (Lin *et al,* 1990). This fraction 1 antigen proved to have good sensitivity and specificity for specific Igs, IgG and IgA by ELISA, but needs further purification to exclude the possibility of reactivity to other parasites.

The criteria of cure in clonorchiasis therapy is the disappearance of the eggs in stool examination for two months after treatment with praziquantel. According to the present results, the use of praziquantel (25 mg/kg body weight in one dose) in the treatment of clonorchiasis shows good therapeutic response. The follow-up survey after successful treatment demonstrated that specific Igs and IgG levels decreased significantly in the 3rd month and IgA levels decreased significantly in the 1st month. Our previous report showed that sera specific Igs, IgG and IgA correlated to the intensity of infection as expressed by EPG, and increased significantly before treatment (Lin *et al,* 1990) IgG and IgA after treatment with praziquantel showed a marked decrease. The significantly higher levels of IgG antibody compared with the control group, strongly implied a possible role in the protection of the host. In schistosomiasis, IgG plays an antibody-dependent and cell-mediated cytotoxicity (ADCC) effector binding activity to the surface antigen of the challenging schistosomula. ADCC reactions on schistosomula were described experimentally with IgG antibody, eosinophils and/or neutrophils in patients or infected animals (Dean *et al,* 1974; Butterworth *et al,* 1977; Capron *et al,* 1978). The sera of specific IgA correlates with egg output and may reflect the greater efficiency of the egg's passage through the intestine, leading to stimulation of the mucosal-related immune system. We may deduce that *C. sinensis* infection will stimulate Igs, IgG and IgA antibody levels in correlation to EPG, causing significantly higher levels before treatment, and that the levels diminish after treatment because of the disappearance of the worms.

In contrast, no significant decrease in IgM was observed. IgM is always present in most acute infective stages. It is possible that our patients were not in an acute stage and, therefore, showed no difference over long term observation. The IgE antibody has been shown to indicate IgE-dependent killing with eosinophils *in vitro* in human schistosomiasis (Capron *et al,* 1984). The binding of IgE to macrophage also demonstrated its activation in the killing of schistosomula. However, in our results, the sera of IgE showed no significant change after treatment. This requires further investigation.

From the point of view of medical practice, serological reactions offer important information useful in assessing whether there has been complete cure of parasitic infection or not. Antibody levels declined significantly in completely cured patients. The present results indicate that detection of specific Igs, specifically IgG and IgA, will be useful to verify the reappearance of eggs after treatment due to relapse or reinfection. It is possible for indicator detection of serum specific Igs, IgG or IgA to be used in combination with stool examination, for evaluation of the effect of praziquantel and the completeness of the clonorchiasis cure. The information from these tests seems to provide one of the most reliable criteria of cure. In addition, sera of specific Igs containing IgG, IgM and IgA could be applied to epidemiological surveys. Using detection of specific Igs chronically and acutely infected individuals could be examined simultaneously.

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