PURIFICATION OF A SPECIFIC IMMUNODIAGNOSTIC *PARASTRONGYLUS CANTONENSIS* ANTIGEN BY ELECTROELUTION FROM SDS-POLYACRYLAMIDE GELS

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Abstract. A 31-kDa glycoprotein antigen was purified by electrophoresing the crude extract of *Parastrongylus cantonensis* adult worms in a 12% SDS-polyacrylamide gel, identifying the 31-kDa component with prestained molecular weight standards, cutting the desired gel strip, and then isolating it by electroelution. Antigen fraction of 31 kDa was re-electrophoresed, transferred to a nitrocellulose membrane and found to be reactive with only the sera from patients with parastrongyliasis. No reactive band was observed with the sera from other related parasitic infections, *eg*, gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis and malaria, and the normal healthy control sera. This antigen fraction isolated showed 100% sensitivity and 100% specificity in the enzyme-linked immunosorbent assay (ELISA) for the detection of 31-kDa specific antibody in the sera from patients with parastrongyliasis. The *P. cantonensis* antigen of 31 kDa has been obtained by this means with a high degree of purity and applied successfully in conventional ELISA for the specific immunodiagnosis of human parastrongyliasis.

INTRODUCTION

The purification of *Parastrongylus* (=*Angiostrongylus*) *cantonensis* antigens in order to improve the specificity of immunological tests for human parastrongyliasis (=angiostrongyliasis) has been reported previously. Chen (1975) and Sato *et al* (1975) obtained an antigenic fraction of *P. cantonensis* with good IHA activity by DEAE-cellulose chromatography. Another *P. cantonensis* fraction containing most of the antigenicity for the IHA test was obtained using the Sephadex G-200 chromatography (Kamiya *et al*, 1973). Welch *et al* (1980) used an affinity chromatography purified *P. cantonensis* antigen on a novel IFAT and was shown to be more sensitive than the ordinary IFAT using worm sections or beads coated with crude antigens. Ko *et al* (1984) and Kum and Ko (1985, 1986) obtained significantly high absorbance values for diagnostic purpose in ELISA by using purified *P. cantonensis* adult worm fractions having pI values of 3.7, 4.0 and 4.45 isolated by preparative flatbed isoelectric focusing. Chen (1986) also showed in ELISA determination that a Sephacryl S-300 purified antigen of *P. cantonensis* was more sensitive than crude antigens. More recently, an eluted fraction obtained by immuno-affinity chromatography was also proved to be highly sensitive in ELISA for serum antibodies to *P. cantonensis* (Yen and Chen, 1991).

A specific, glycoprotein antigen for immunodiagnosis of parastrongyliasis in mans was recently identified and characterized. This diagnostic antigen was a peptide of 31 kDa [(determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)] under reducing and non-reducing conditions), and the antigenic activity of this molecule was apparently maintained after sodium periodate treatment (Eamsobhana *et al*, 1997; 1998).

The present work was designed to isolate a single target-specific immunodiagnostic antigen of 31 kDa, using SDS-PAGE followed by electroelution. This isolated antigen fraction was then used in immunoblotting and

ELISA for the detection of a 31-kDa serum antibody from patients with parastrongyliasis. Sera from patients infected with other parasitic infections and normal healthy individuals were also examined in order to determine the specificity of this purified fraction.

MATERIALS AND METHODS

Parasites and parasite antigens

P. cantonensis Thailand strain was originally obtained from the Faculty of Tropical Medicine, Mahidol University and has been propagated in our laboratory by cycling it through Wistar strain rats and planorbid snails, *Biomphalaria glabrata*. Adult worms were obtained from the pulmonary arteries of the rats that were infected for at least 6 weeks with *P. cantonensis* infective larvae collected from experimentally infected snails. Female worms were separated, washed and then homogenized in a small volume of normal saline with a glass tissue grinder. The suspension was then sonicated and left overnight at 4ºC to allow elution of antigens. Soluble antigen was obtained as the supernatant after centrifugation at 5,000 rpm for 15 minutes. The protein content of the extract was determined using a protein assay kit II (Bio-Rad Laboratories, USA).

Serum samples

Serum samples were obtained from five patients with parasitologically proven parastrongyliasis (three with cerebral parastrongyliasis from whom *P. cantonensis* larvae were recovered from the CSF; other two had ocular parastrongyliasis from whom immature *P. cantonensis* worms were recovered from their eye chambers) and five patients with presumptive parastrongyliasis. The latter group was diagnosed as parastrongyliasis based on clinical symptoms and history of exposure to infection, as well as having high antibody titers as detected by ELISA.

Antigen cross-reactivity studies were carried out on sera from 10 patients each with gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis, and malaria. All these cases were positive by parasitologic and/or serologic tests for a specific parasite or its products. Two of the patients with gnathostomiasis and all patients with filariasis, paragonimiasis and malaria were diagnosed parasitologically. The ten cases of toxocariasis were serologically positive on ELISA using excretory-secretory antigens of the second-stage larvae of *T. canis*, while the patients with cysticercosis were diagnosed pathologically as subcutaneous cysticerci. The normal control group of sera were obtained from 50 healthy adults who were negative for any parasitic infection at the time of blood collection. All serum samples were kept at -20ºC until use.

Isolation of antigen from sodium dodecyl sulphate-polyacrylamide gel by electroelution

SDS-PAGE was carried out according to the method of Laemmli (1970). Briefly, 1.5-mm single trough of a12% separating gel was loaded with about 2.0 mg of the female worm extract of *P. cantonensis*. Individual slots in the same gel were used to electrophorese the high and low molecular weight standards. Once the gel ran its full length, strips with the molecular standards were cut and rapidly stained with Coomassie brilliant blue R 250 to determine the region where the 31-kDa antigen of interest would be according to approximate molecular weight. A region that included the 31-kDa targeted antigen was cut into strip and the gel pieces were electroeluted in an electro-eluter (Model 422, Bio-Rad) using a clear membrane cap at a molecular weight cut off of 12,000-15,000 daltons. Elution was done at 10 mA/glass tube constant current for 3 hours. Electroeluated protein was desalted and concentrated by ultrafiltration using Centricon YM-10 (Millipore Corporation, USA). Protein content estimated using Protein Assay Kit II (Bio-Rad).

Electrophoresis and immunoblotting

SDS-PAGE was carried out similarly to the procedure described above. In brief, crude

antigenic extract of *P. cantonensis* (10 μg of proteins per lane) or eluted 31-kDa antigen fraction of *P. cantonensis* (5 μg of proteins per lane) was separated on a 12% reducing SDS-polyacrylamide slab gel. After electrophoresis, the resolved polypeptide bands were either revealed by staining with Coomassie brilliant blue R 250 or electrophoretically transferred from the gel to a nitrocellulose membrane for immunoblotting, which was conducted as described by Towbin *et al* (1979). The non-specific binding sites on the membrane were blocked by soaking in a solution of 5% skimmed milk in phosphate-buffered saline (PBS), pH 7.4 for 1 hour. The membrane was then incubated with test serum, which was diluted 1: 200 in 1% bovine serum albumin (BSA) in PBS, pH 7.4 overnight at 4ºC. After washing thoroughly the membrane was then reacted with horseradish peroxidase-conjugated rabbit anti-human immunoglobulins (Dakopatt, Denmark) at a dilution of 1: 1,000 in PBS, pH 7.4 for 1 hour at room temperature followed by washing. The bound antigen-antibody complexes were visualized by adding the chromogenic substrate solution containing 30 mg of 4-chloro-1-naphthol (Pierce Chemical Company, USA) in 10 ml of absolute methanol mixed with 100 ml of 30% H_2O_2 in 100 ml of PBS, pH 7.4. The blots were rinsed in distilled water, air-dried and photographed.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed essentially as described previously (Eamsobhana *et al*, 1999). Eluted 31-kDa antigen fraction of *P. cantonensis* at a concentration of 1 μg/ml in carbonate buffer, pH 9.6 was incubated in wells of microtiter plate (Nunc, Denmark). Antigen was incubated 2 hours at 37ºC and then overnight at 4ºC. Sera and the horseradish peroxidase-conjugated rabbit anti-human immunoglobulins (Dakopatt, Denmark) were used at 1:100 and 1:1,000 dilutions, respectively, and both were incubated for 2 hours at 37ºC. The substrate used was o-phenylenediamine and was incubated for 30 minutes at room temperature in the dark. The enzymatic reaction was stopped with 50 μl of 2.5N sulphuric acid and the plate read spectrophotometrically at 492 nm with an ELISA reader (SLT Labinstrument, Australia). Final volumes of 100 μl per well were used for antigen, sera, conjugate, and substrate.

The optimal concentration of the antigen and the optimal dilution for patient's serum and conjugate were pre-determined using a chequerboard titration. For each test, a negative, a positive and a PBS-Tween controls were included.

A result was considered positive if the OD value exceeded the mean OD+3SD of the values obtained with the 50 negative sera.

RESULTS

A 31-kDa glycoprotein was successfully isolated from the crude female worm extract of *P. cantonensis*. Upon analysis by Coomassie brilliant blue stained gel, this fraction appeared as a single band with minor impurities (Fig 1).

Immunoblot analysis of the isolated 31-kDa antigen of *P. cantonensis* against representative human sera with confirmed parastrongyliasis and other related parasitic infections is shown in Fig 2. Sera from *P. cantonensis*-infected patients reacted strongly with the isolated antigen fraction to show a single band of 31 kDa (Lane A). On the other hand, this 31-kDa antigen was not recognized by sera from patients with gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis and malaria (Lanes B-G) and sera from normal healthy individuals (Lane I).

When ELISA was applied as a diagnostic test, the mean OD value ($\bar{X}$±SD) of the normal group was 0.061±0.019. The mean plus three standard deviation OD value of the healthy group sera was then taken as the cut-off value, OD>0.118 indicated positive results. As shown in Fig 3, all the serum samples from parastrongyliasis cases (10/10) were positive in the ELISA. No cross-reaction was

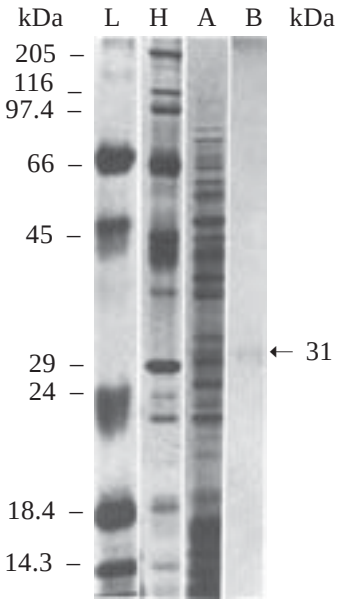

Fig 1–SDS-PAGE and Coomassie brilliant blue R 250 staining patterns of crude adult female worm extract (A) and an electroeluted 31-kDa antigen (B) of *P. cantonensis*. L and H are low and high molecular weight standards (Sigma). The position of the diagnostic antigen is indicated by the arrow.

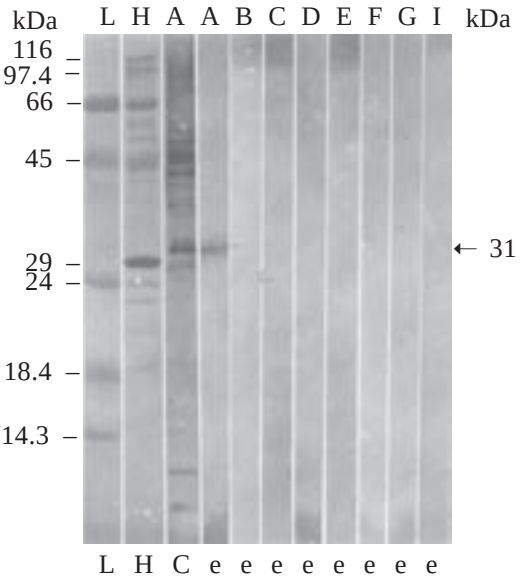

Fig 2–Immunoblot analysis showing reactivity of serum samples from patients with parastrongyliasis (A), gnathostomiasis (B), toxocariasis (C), filariasis (D), paragonimiasis (E), cysticercosis (F) and malaria (G), and normal control serum (I) against an electroeluted 31-kDa *P. cantonensis* antigen (e) and crude female worm extract (c). L and H are low and high molecular weight standards (Sigma). The position of the diagnostic antigen is indicated by the arrow.

found with serum samples from other parasitic infections. Sera from patients with gnathostomiasis, toxocariasis, filariasis, paragonimiasis, cysticercosis and malaria and normal parasite-free individuals were all negative. The sensitivity and specificity of the ELISA using this electroeluted, purified 31-kDa antigen were both 100%.

Of the 12 mg protein of the crude extract of *P. cantonensis* initially applied for electrophoresing in a total of six SDS-polyacrylamide gels, after electroelution, about 0.49 mg protein (4.08% yield) of the eluate was obtained.

DISCUSSION

The antigens used in most immunological studies of human parastrongyliasis have been prepared from the adult worms of *P. cantonensis*. Because of the fairly broad cross-reactivities of the crude extracts prepared from adult *P. cantonensis*, it is necessary to generate a specific antigen. Otherwise, immunological techniques would have a restricted application. An antigen of 31 kDa was one of the most prominent in all the sera tested for human parastrongyliasis revealed by immunoblot analysis (Eamsobhana *et al*, 1997). This 31 kDa protein was targeted for isolation because it did not react in immunoblotting with the heterologous sera tested (Eamsobhana *et al*, 1997).

The present study attempts to purify this diagnostic antigen from the crude worm extract after electrophoresing in a SDS-gel using an electro-eluter. A fraction of protein having

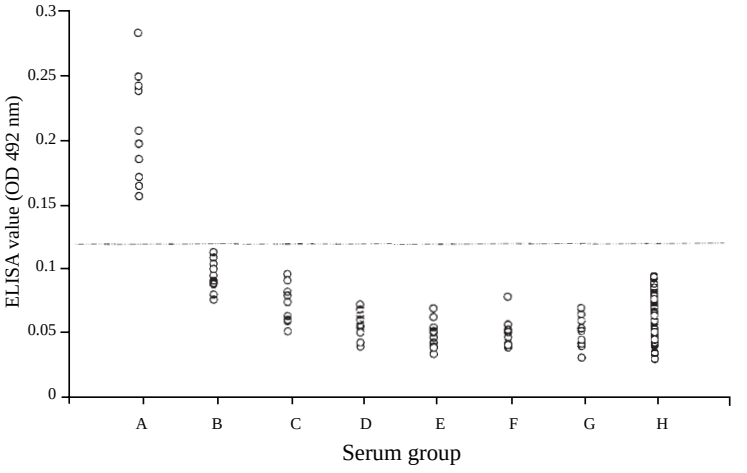

Fig 3–Distribution of optical density values in ELISA for detection of serum antibody using electroeluted 31-kDa *P. cantonensis* antigen in 10 patients each with parastrongyliasis (A), gnathostomiasis (B), toxocariasis (C), filariasis (D), paragonimiasis (E), cysticercosis (F), and malaria (G), and in 50 normal healthy individuals (H). The dotted line shows the cut-off value.

high reactivity with a 31-kDa *P. cantonensis* specific antibody was isolated. Electrophoresis under reducing conditions revealed this electroeluted fraction as a single protein band. When the eluted 31-kDa antigen of *P. cantonensis* was used in the immunoblot to identify the specific antibody formation in patients with parasitologically confirmed parastrongyliasis, the antigenic band of 31 kDa was prominently recognized by all the parastrongyliasis sera tested. This antigen appears to have good purity and specificity, as it is not recognized by antibodies to other related parasites (*eg*, *Gnathostoma spinigerum*, *Toxocara* spp, *Wuchereria bancrofti*, *Paragonimus heterotremus*, *Taenia* spp and *Plasmodium falciparum*). This is also confirmed by the reactivity shown in ELISA when the electroeluted 31-kDa antigen was used in the assay. This isolated antigen was able to clearly discriminate between infected sera from parastrongyliasis and other clinically related parasitic infections and normal control.

Taken together, the present results indicate that a specific 31-kDa antigen was successfully isolated from the complex crude extract of adult *P. cantonensis* by adopting a system of electroelution. This antigen of 31 kDa has been isolated to homogeneity by electroeluting the band from SDS-PAGE gels. Results obtained in six SDS-PAGE gels suggest reproducibility and maintenance of reactivity using this isolation procedure. Clearly, electroelution is a powerful method to purify immunodiagnostic antigen in small amounts. However, it is obvious that one of the obstacles in the purification of antigens from the crude extracts of helminths is the difficulty in obtaining a substantial working quantity of the parasite material for immunodiagnosis. In the present study, an average of 4.08% yield of the purified material was obtained, thus indicating the system used may have some practical applications. At present, the optimal parameters for the use of this described purified reagent of *P. cantonensis* in dot-blot ELISA, a more rapid and simple test, have been determined and the test has been evaluated.

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