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Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Circulating Antigen in *Schistosoma japonicum* Infections

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The study of free circulating antigen (CA) in schistosome infections was first published by Berggren and Weller (1967), who detected the antigen in plasma of mice heavily infected with *Schistosoma mansoni*. Detailed studies showed that the property of CA was a negatively charged high molecular (more than 100,000 MW) proteoglycan which was trichloroacetic acid-soluble and heat-stable (Nash *et al.*, 1974; Deelder *et al.*, 1976; Nash *et al.*, 1977). This antigen was found in the epithelial cells of the gut of worm and released into the host circulation (Nash *et al.*, 1974). In *S. japonicum* infections, CA could be also demonstrated in sera of rabbits, mice and chimpanzees (Hirata and Akusawa, 1974; Hirata, 1976; Hillyer, 1976).

In the present study, for detecting a low level of antigen sensitively, sandwich enzyme-linked immunosorbent assay (ELISA) using anti-worm rabbit IgG was attempted to demonstrate CA in sera of experimental animals and humans infected with *S. japonicum*. Counter immunoelectrophoresis (CIE) was performed as a reference test for ELISA.

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Materials and Methods

Preparation of anti-worm rabbit IgG: The adult worms of *S. japonicum* (Yamanashi strain) were collected from infected mice (DDD). Rabbits were then hyperimmunized with the worm homogenate mixed with Freund's complete adjuvant. Immunized rabbit serum was absorbed with normal mouse serum because worm used for immunization contained mouse serum components in its body. Anti-worm antibody was purified from the absorbed rabbit serum by an immunoadsorbent column using CNBr-activated Sepharose 4B (Pharmacia) coupled with soluble worm antigen (30 mg Folin-reactive protein/5 ml gel). Thus prepared antibody was further passed through a DEAE cellulose (Brown) column to obtain IgG fraction. The final fraction which was used for ELISA and CIE showed 7–9 precipitin bands against the worm antigen by immunoelectrophoresis.

Sandwich enzyme-linked immunosorbent assay (ELISA): ELISA for detection of CA in serum was a modification of the method established for the purpose of detecting the antibody specific to the worm

and the egg of *S. japonicum* (Tanaka *et al.*, 1979; Nakao *et al.*, 1981; Matsuda *et al.*, 1981). Purified anti-worm rabbit IgG was labeled with horseradish peroxidase (type VI, Sigma) by the method of Nakane and Kawaoi (1974). The assay was performed in 0.2 ml/well on micro-ELISA plate (M129A, Dynatech). Wells of the plate were coated with anti-worm rabbit IgG diluted at 20 μg /ml with carbonate buffer (0.05 M, pH 9.6) at 4 C overnight. The wells were washed three times for 15 minutes with phosphate buffered saline (PBS, 0.15 M, pH 7.2) containing 0.05% tween 20 and 0.05% sodium azide after each procedure of coating with the IgG, binding the antigen and binding the labeled IgG. Test serum and the labeled IgG were diluted with PBS containing 1% bovine serum albumin (fraction V, Sigma) and 0.05% tween 20. Test serum diluted at 1:2 was reacted with the IgG coated on the well wall. After washing, the labeled IgG diluted at 70–140 μg/ml was applied. The plate was incubated at 37 C for 60 minutes in a moisture box for each reaction. O-phenylenediamine (OPD, Eastman Kodak) was used as a substrate for the enzyme. OPD was dissolved in methanol (10 mg/ml), and 0.5 ml of OPD solution together with 0.05 ml of 3% H_2O_2 was mixed with 49.5 ml of distilled water immediately before use. The final substrate reaction proceeded in a dark place at room temperature for 30 minutes, then stopped with a drop of 8 N H_2SO_4/well. Absorbance of the substrate after the reaction was measured by a plate reader (MTP12, Corona) at 500 nm.

Counter immunoelectrophoresis (CIE): CIE was carried out on a glass plate (72× 107 mm) covered with 13 ml of 1% agar (Difco) in barbital buffer (pH 8.6, 0.05 ionic strength) according to the method of Hirata *et al.* (1977). Wells were punched out using a 3 mm metal tube. The distance between anodic and cathodic wells was 4 mm. The anodic wells were filled with 10 μl of anti-worm rabbit IgG (4 mg/ml) and the cathodic wells were with 10 μl of undiluted test sera. Electrophoresis ran at room temperature with the same buffer at 60 volts/72 mm for 45 minutes. The reaction on the plate was read immediately after run.

Results

Specificity and sensitivity of ELISA: Using the worm antigens extracted with PBS from adult *S. japonicum* and *Fasciola hepatica,* the specificity and the sensitivity of the reaction of sandwich ELISA were examined with different amounts of these antigens. Since high reaction was obtained only with *S. japonicum* antigen in this method, it was thought that this method was highly specific for detecting CA derived from the worm of *S. japonicum* (Fig. 1). The worm antigen of *S. japonicum* at 0.5 μg/ml was an enough level to be detectable by this method.

The ELISA value, the percentage of absorbance by CA in test serum to that by

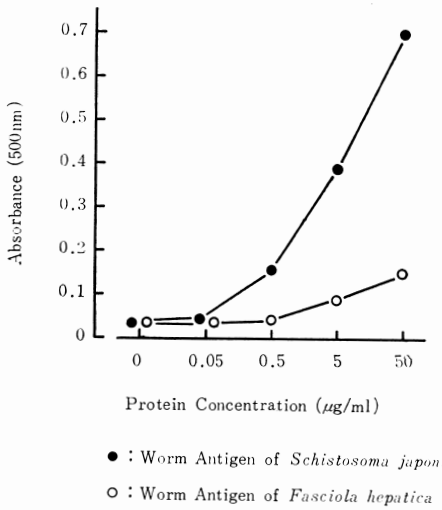


Fig. 1 Reactions in sandwich ELISA with prepared anti-worm rabbit IgG and its conjugate with peroxidase to examine the specificity and the sensitivity in detecting *S. japonicum* antigen.

the worm antigen at 5 μg/ml was utilized to show CA level in test serum according to the method of calculation by Carlier *et al.* (1980) as follows:

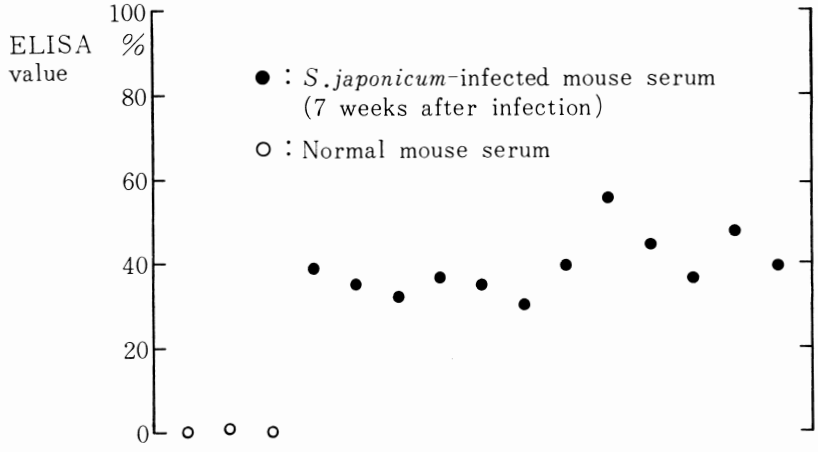
$$\text{ELISA value (\%)} = \frac{\text{Absorbance by test serum} - \text{Absorbance by normal serum}}{\text{Absorbance by normal serum containing worm antigen at 5 } \mu\text{g/ml} - \text{Absorbance by normal serum}} \times 100$$

The ELISA value at 20% was conventionally determined as the minimum ELISA value for CA positive.

CA in serum of infected mouse: Twelve mice (DDD) were infected with about 70 cercariae by subcutaneous injection and sacrificed 7 weeks after infection for collecting serum samples and counting the number of adult worms. CA was demonstrated in all sera of these 12 infected mice but not in sera of 3 normal mice by ELISA and CIE (Fig. 2). The ELISA values in infected mice were distributed in a narrow range from 30.0 to 55.7% irrespective of the worm burden.

CA in serum of infected rabbit: Shaved skins of 5 rabbits were exposed to cercariae quantitatively to produce 3 light infections with 10, 20 and 70 cercariae each and 2 heavy infections with 800 cercariae. Serum samples were collected weekly from each rabbit. Heavily infected ones died 10 weeks after infection. CA was demonstrated only in sera of these 2 heavily infected rabbits by ELISA and CIE from 6 weeks after infection until before die (Fig. 3). It was observed that the elevation of detectable level of CA in rabbits was dependent on the worm burden in this experiment.

CA in serum of human patient: An attempt was made to detect CA in sera of human patients. Sera from 40 proven patients of schistosomiasis japonica in Leyte, Philippines were examined for CA



Results of CIE	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
Number of worms	0	0	0	16	18	20	20	24	28	30	38	42	44	48	52

+ : Positive − : Negative

Fig. 2 Results of ELISA and CIE in infected and normal mouse sera.

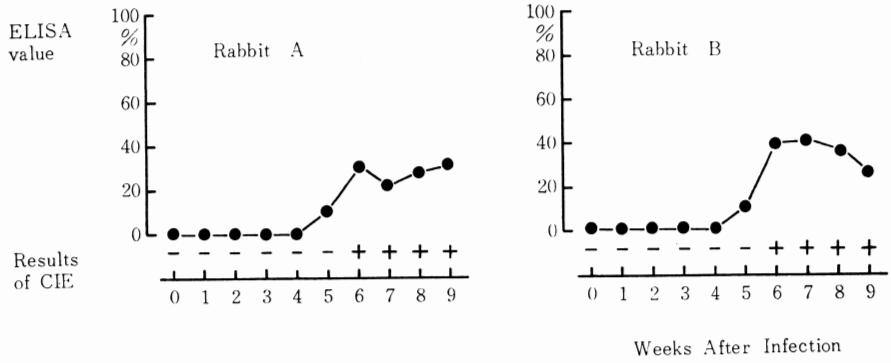


Fig. 3 Detection of circulating antigen in heavily infected rabbit sera by ELISA and CIE.

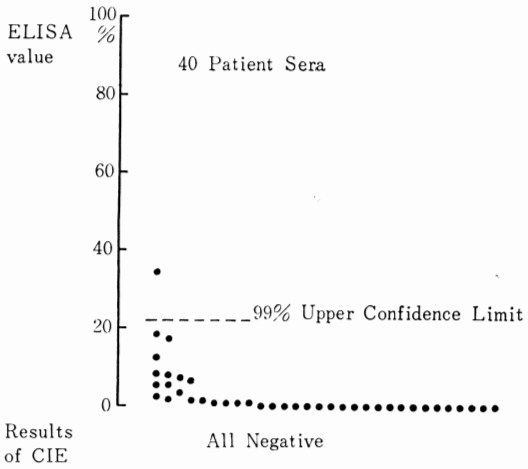


Fig. 4 Detection of circulating antigen in human patient sera.

by ELISA and CIE. All sera were negative by CIE. Nevertheless, one serum out of 40 samples showed high ELISA value of 34.0% which was more than the upper confidence limit (Fig. 4).

Discussion

Enzyme-linked immunosorbent assay (EL ISA) has an advantage of high sensitivity as in nearly the same level as radioimmunoassay (RIA), and its procedure is safe and economical in comparison with RIA. Improved ELISA using micro-plates is capable of testing many serum samples in a short time. In parasitological field, ELISA has been used as a diagnostic tool for detecting specific antibodies in malaria, trypanosomiasis, toxoplasmosis, echinococcosis, schistosomiasis and trichinosis. ELISA has not generally utilized for detecting antigens from parasites.

In this investigation, the circulating antigen (CA) derived from the worm of *S. japonicum* was detected in sera of infected mice and rabbits by sandwich ELISA. The sensitivity of sandwich ELISA for detecting CA was almost the same as that of counter immunoelectrophoresis (CIE). Sera showing positive for CIE had the higher ELISA values than 20% which was conventionally determined as the minimum ELISA value for CA positive.

It was observed that the frequency of occurrence of CA and its concentration were relatively high in mice comparing with those in rabbits. It seemed that the occurrence of CA was closely dependent on the blood volume of the host, the relative worm burden to the size of host, the duration of infection and the immunological response of the host. Hirata (1981) reported that CA level was dependent on the female worm in *S. japonicum* infections.

In human patients, ELISA for detecting

CA was not a practical tool for diagnosis and case detection. The detection of antibody against CA would be more useful for this purpose.

Summary

Sandwich enzyme-linked immunosorbent assay (ELISA) to detect free circulating antigen (CA) in *Schistosoma japonicum* infections was successfully made. Anti-worm rabbit IgG and that labeled with peroxidase were used for the sandwich method. Counter immunoelectrophoresis (CIE) was performed as a reference test for ELISA. In experimental animals, CA was found in sera of infected mice and heavily infected rabbits. The results of ELISA coincided with those of CIE. In human patient sera, CA could not be detected by CIE but only one serum out of 40 samples showed a high antigenic level by ELISA.

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酵素抗体法（ELISA）による日本住血吸虫由来の循環抗原の検出

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日本住血吸虫感染時に宿主の血清中に現れるといわれる虫体由来の循環抗原を酵素抗体法（ELISA）により検出した．また，免疫電気向流法（CIE）で循環抗原を検出し，ELISAの結果と比較した．

抗成虫抗体をウサギにより作成し，特異IgG分画を得て，ELISAとCIEに用いた．ELISAにはペルオキシダーゼ標識抗成虫抗体を作成し，マイクロタイタープレートで固相サンドイッチ法を行った．CIEには高電気浸透の1％アガーを用いた．

感染後7週のマウス血清では感染虫体数にかかわらずに12例中全例に循環抗原を証明できた．感染ウサギ血清では800セルカリア感染のもので感染後6週以後9週まで（10週目に死亡）循環抗原を証明できたが，10，20，70セルカリア感染のものではできなかった．ELISAとCIEの抗原検出感度はほぼ同じであった．フィリピン・レイテ島における本症患者血清40例について循環抗原を検索し，CIEではすべて陰性であったが，ELISAでは1例に高値を得た．

本症の免疫血清診断では，循環抗原検出よりも，特異抗体検出が重要であると思われた．