

Detection of Circulating Excretory Secretory Antigens in Human Fascioliasis by Sandwich Enzyme-Linked Immunosorbent Assay

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We developed a sandwich enzyme-linked immunosorbent assay to detect circulating parasite antigen in humans with fascioliasis. The assay uses antibodies against *Fasciola hepatica* excretory secretory (ES) antigens. A monoclonal antibody was used to capture circulating ES antigens, and a human polyclonal antibody peroxidase conjugate was used to identify circulating ES antigens. Optimal dilutions of all reagents were determined by block titration. The antigen concentration in sera from patients was estimated by comparing the optical density at 492 nm of test sera with a standard curve. All of the serum samples from 25 patients with parasitological evidence of fascioliasis had a detectable antigen concentration (more than 10 ng/ml). None of the serum samples from 80 patients infected with parasites other than *F. hepatica* showed a positive reaction, suggesting the absence of cross-reactions in this assay.

There is a need to improve the diagnosis of human fascioliasis. Parasitologic diagnosis may be difficult because of intermittent shedding of parasite eggs in stools or in patients with recent infections, since eggs are found in fecal samples only after 6 to 8 weeks. Serological tests for antibodies are of limited diagnostic value because antibody titers persist after the patients have been cured. In addition, it is possible that patients with recent infection have a negative test, a phenomenon demonstrated in experimental animals during the first 1 to 4 weeks of infection (5, 6).

The detection of circulating parasite antigens was evaluated recently as an alternative procedure for the diagnosis of schistosomiasis (3, 12, 16), filariasis (7, 11, 15), toxocarosis (13), and toxoplasmosis (1, 2). Only a few attempts to detect circulating antigens in patients with fascioliasis have been reported (8, 9). We report here the use of a sandwich enzyme-linked immunosorbent assay (ELISA) technique to detect circulating excretory secretory (ES) antigens of *Fasciola hepatica* by use of a monoclonal antibody.

MATERIALS AND METHODS

Human sera. Single serum samples from 25 patients infected with *F. hepatica* and 80 patients infected with parasitologically proved infections other than *F. hepatica* were studied. The 80 patients with other infections included 20 patients infected with *Entamoeba histolytica*, 14 patients infected with *Ascaris lumbricoides*, 12 patients infected with *Trichuris trichiura*, 6 patients infected with *Schistosoma mansoni*, 4 patients infected with *Schistosoma haematobium*, 10 patients infected with *Giardia lamblia*, 6 patients infected with *Wuchereria bancrofti*, 4 patients infected with *Loa loa*, and 4 patients infected with *Mansonella perstans*. A further 100 healthy subjects with repeated negative results on examination of stool specimens were used as negative controls. The sera from patients and controls were stored frozen at -20°C until use.

ES antigens. Live intact *F. hepatica* adult worms were obtained from bovine livers at a local abattoir, as described previously (4). They were washed four times at room tem-

perature with 0.9% sodium chloride to remove all traces of blood and bile and incubated at 37°C in RPMI 1640 medium with antibiotics. After 24 h, the medium was centrifuged ($1,500 \times g$ for 10 min at 4°C). The supernatant was collected and concentrated by using a high-flow membrane filter (UM-10; Amicon Corp.). Protein concentration was determined by the method of Lowry et al. (10). The antigens were stored at -20°C until use.

Development of MAbs. Female BALB/c mice (age, 6 weeks) were sensitized by five subcutaneous injections of $100 \mu\text{g}$ of *F. hepatica* ES antigens at weekly intervals. For the first injection Freund complete adjuvant was used, while for the other four injections Freund incomplete adjuvant was used. Three days after the last injection, the spleens were removed and the splenic lymphocytes were fused with cultured P3 \times G3Ag8-6.5.3 myeloma cells (at a ratio of 10:1) by using 42% (wt/vol) polyethylene glycol (M_r , 3,000; Sigma Chemical Co.). Spleen \times myeloma hybrids were selected with hypoxanthine-aminopterin-thymidine medium in microtiter plates. After 2 weeks of culture, the medium from each well was screened by ELISA for the presence of antibody against ES antigens as described previously (4), which was modified by the use of horseradish peroxidase-labeled goat anti-mouse immunoglobulin (IgG; Bio-Rad Laboratories). After 2 weeks, ascitic fluids containing 1 to 10 mg of total protein per ml were harvested from the peritoneal cavity. Mouse IgG monoclonal antibody (MAb) was then isolated from ascitic fluids by a single-step method by using a mono-Q-HR 5/5 column in a fast-protein liquid chromatography system (Pharmacia, Uppsala, Sweden).

Preparation of enzyme conjugate. Sera from patients infected with *F. hepatica* showing high antibody titer ($>1:50,000$) by ELISA against ES antigens (4) were pooled. The immunoglobulin fraction was prepared by using 50% ammonium sulfate precipitation. The precipitated immunoglobulin fraction was resuspended in a small volume of 0.01 M phosphate-buffered saline (PBS; pH 7.2), dialyzed against an excess of PBS, and passed through a prepacked mono-Q-HR 5/5 column (Pharmacia). The protein in the unabsorbed fraction was confirmed as IgG by Ouchterlony double diffusion against rabbit anti-human IgG serum. IgG was concentrated to 10 mg/ml by using a high-flow membrane filter

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(UM-100; Amicon Corp.) and conjugated to horseradish peroxidase (type VI; Sigma) by the method of Nakane and Kawaoi (14).

Circulating antigens. Levels of *F. hepatica* ES antigens in sera of patients were estimated by sandwich ELISA by using disposable polyvinylchloride microtiter plates (Falcon, Becton Dickinson and Co.). Optimal dilutions of the MAb and conjugate were found by a checkerboard titration in which serial dilutions of MAb (from 0.25 to 2.0 $\mu\text{g/ml}$) and anti-*F. hepatica* IgG-peroxidase conjugate (from 1:1,000 to 1:3,000) were tested simultaneously by using a negative and a positive control serum sample in each plate. For each layer, 100- μl portions were used. Plates were sensitized overnight at 4°C by the addition of MAb diluted in 0.1 M carbonate buffer (pH 9.6). They were washed three times with PBS containing 0.05% Tween 20 (PBS-T20), and unbound sites in the wells were blocked with 5% bovine serum albumin diluted in carbonate buffer. After incubation for 1 h at 37°C, the plates were emptied by suction. Undiluted human serum was added and the plates were incubated overnight at 4°C, after which the plates were washed six times with PBS-T20. Anti-*F. hepatica* IgG-peroxidase conjugate was added and diluted in PBS-T20–10% fetal bovine serum, and the plates were incubated for 2 h at room temperature. After another washing, the substrate *o*-phenylenediamine dihydrochloride (Sigma) was added, and the plates were incubated for 30 min in the dark at room temperature. The enzyme reaction was stopped with 50 μl of 12.5% H_2SO_4 . The plates were read at 492 nm by using a Titertek Multiskan ELISA reader. In each plate three different controls were used: (i) a negative control serum sample that yielded negative results on initial and repeated testing, (ii) a positive control serum sample prepared by mixing 156 ng of ES antigen protein with 1 ml of negative control serum, and (iii) a conjugate control in which PBS-T20 was substituted for the serum.

Sensitivity of the antigen detection assay. A standard curve was generated by spiking negative control serum with known quantities of ES antigens (from 5 to 625 ng/ml). The optical density at 492 nm (OD_{492}) of spiked negative control serum was plotted against its known antigen protein concentration. The standard curve had an r^2 of 0.963. By use of the standard curve, ODs of sera from patients infected with *F. hepatica* were transformed into antigen concentrations.

Statistical analysis. Each assay was repeated four times, and the tests were run on four different days. The results were expressed as the mean absorbance value (mean OD) for each determination. Test results were considered positive if the OD exceeded the mean \pm 2 standard deviations (SDs) of the OD obtained with the known negative control sera. Statistical analysis of the data was done by using Student's *t* test.

RESULTS AND DISCUSSION

Two criteria were used to select the MAb used in the present study: high signal-to-background ratio and apparent reactivity with distinct antigenic epitopes. Thus, a large number of available MAbs were screened for their ability to detect small amounts of ES antigens. Under optimal assay conditions, MAb ES78 (mouse IgG2a) was selected for its ability to detect at least 10 ng of ES antigen added to normal human serum.

The optimal dilution of all reagents was determined by checkerboard titration by using different dilutions of MAb ES78 and conjugate. Results were plotted by using the signal-to-noise ratio against the ES78 concentration. The

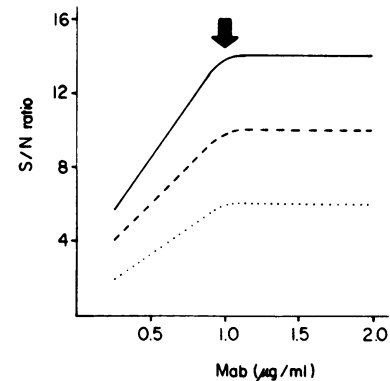


FIG. 1. Checkerboard titration to determine optimal MAb (ES78) and conjugate dilution. The signal-to-noise (S/N) ratio (ratio of observed OD reading of positive control serum to the observed OD reading of negative control serum) versus ES78 concentration was tested at three conjugate dilutions: —, 1:1,000; ----, 1:2,000; ·····, 1:3,000. The arrow points to the optimal dilutions.

signal-to-noise ratio was the ratio of the observed OD reading of positive control serum to the observed OD reading of negative control serum to each ES78 concentration. The data obtained showed that the 1:1,000 conjugate dilution offers the best signal-to-noise ratio. The ES78 concentrations of 0.25 and 0.5 μg of protein per ml were inadequate to saturate the wells. The best results were obtained when the plate was coated with 1.0 $\mu\text{g/ml}$ (Fig. 1). Negative/positive discrimination levels for test results were determined by using 100 negative serum samples derived from healthy subjects and 80 serum samples derived from patients infected with parasites other than *F. hepatica*. The mean OD of the negative group was 0.15 ± 0.04 (SD). Sera from patients with fascioliasis showed a range of ODs between 0.26 and 0.93, with a mean value of 0.47 ± 0.17 (SD), whereas the OD values in sera from patients with other parasitic infections were between 0.09 and 0.22 ($0.1 < \text{OD} < 0.20$; $0.01 < \text{SD} < 0.03$). There was a significant difference between mean sandwich ELISA values for patients with fascioliasis and those for patients infected with other parasites ($P < 0.001$). Considering the whole nonfascioliasis group (sera from controls and patients infected with other parasites), the MAb-sandwich ELISA was specific for diagnosis of fascioliasis when OD values were greater than $0.15 + 2 \times 0.04$ (>10 ng/ml) (Fig. 2).

To determine approximate amounts of circulating ES antigens in sera of patients with *F. hepatica*, OD_{492} values were compared with the standard curve. The reproducibility and sensitivity of results from four tests run on four different days are shown in Table 1. Sera from all patients had detectable circulating ES antigens (more than 10 ng/ml), 22 of 25 (88%) serum samples had detectable antigen in the range of 14 to 50 ng/ml, and 3 of 25 (12%) serum samples had detectable antigen at a concentration of more than 50 ng/ml. The mean antigen concentration in serum was approximately 32.0 ng/ml. Our patients were considered to have been infected several months prior to clinical and parasitological examination, all having excreted *Fasciola* eggs. It was possible to detect circulating antigen in their sera while the parasite was present and alive in the bile ducts of the host, releasing antigens into the circulation.

Only a few attempts to detect circulating antigens in patients with fascioliasis have been published. Knobloch (8) reported the use of a four-layer ELISA to detect circulating

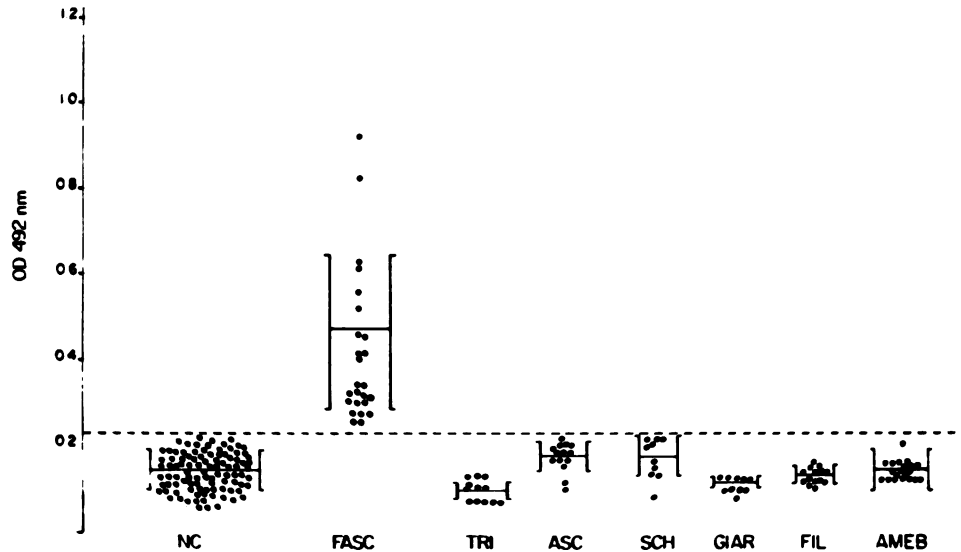


FIG. 2. Specificity of the sandwich ELISA by using MAb ES78 and sera from groups of patients with parasitologically proven infection with fascioliasis (FASC), trichuriasis (TRI), ascariasis (ASC), schistosomiasis (SCH), amebiasis (AMEB), filariasis (FIL), and giardiasis (GIAR), and sera from a negative control group (NC). The statistically significant difference was apparent between the sera of patients with fascioliasis and patients infected with other parasites ($P < 0.001$). -----, cutoff OD.

antigen in 45 patients with parasitologically confirmed chronic fascioliasis by using F(ab)₂ fragments from guinea pig antiserum to soluble extract of adult *F. hepatica* flukes. In that report the sensitivity of the test was very low. Results for sera were recorded as negative when the antigen concentration was <50 ng/ml, and only 9 of 45 serum samples were positive for antigen detection.

TABLE 1. Sensitivity and reproducibility of MAb-sandwich ELISA for the detection of ES antigens of *F. hepatica*^a

Standard curve		Patients infected with <i>F. hepatica</i>		
ES antigen concn (ng/ml)	OD ± SD	No.	OD ± SD	Concn (ng/ml)
625.0	2.66 ± 0.04	1	0.26 ± 0.04	14.2
312.0	1.88 ± 0.03	2	0.28 ± 0.03	19.0
156.0	1.15 ± 0.01	3	0.28 ± 0.03	19.0
78.0	0.73 ± 0.02	4	0.33 ± 0.02	21.8
39.0	0.45 ± 0.04	5	0.30 ± 0.02	20.9
20.0	0.28 ± 0.03	6	0.46 ± 0.03	38.0
10.0	0.23 ± 0.02	7	0.42 ± 0.02	26.6
5.0	0.00	8	0.34 ± 0.03	21.8
		9	0.26 ± 0.05	14.2
Controls	0.00	10	0.30 ± 0.03	20.9
		11	0.73 ± 0.04	77.5
		12	0.56 ± 0.04	41.8
Negative conjugate	0.00	13	0.62 ± 0.03	43.7
		14	0.54 ± 0.02	40.8
		15	0.32 ± 0.03	20.9
		16	0.33 ± 0.02	21.8
		17	0.45 ± 0.02	38.0
		18	0.93 ± 0.03	81.7
		19	0.83 ± 0.02	78.9
		20	0.32 ± 0.04	21.8
		21	0.34 ± 0.03	22.8
		22	0.28 ± 0.05	19.0
		23	0.41 ± 0.05	25.6
		24	0.43 ± 0.03	27.5
		25	0.33 ± 0.03	21.8

^a Results are from four tests run on four different days (mean ± SD of the mean; $y = 0.533 \ln x + 2.589$; $r^2 = 0.963$).

Langley and Hillyer (9) recently reported a two-site ELISA (horseradish peroxidase- or biotin-based antigen capture assay) in which they used anti-*F. hepatica* ES antigen polyclonal antibodies. Using a biotin-based antigen capture assay, they were able to detect 0.25 ng of antigen protein per ml of serum, but in practice, the assay proved to be less sensitive because of high background activity. However, by using the horseradish peroxidase-based capture assay, they were able to detect 25 ng of antigen protein per ml of serum. Results of our assay show an increased sensitivity over those in previously reported tests. This may be due to differences in the assay conditions or to the quality of the antibody used to capture the antigen.

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