Detection of the schistosome circulating cathodic antigen by enzyme immunoassay using biotinylated monoclonal antibodies

N. de Jonge[1*], P. G. Kremsner[2], F. W. Krijger[1], G. Schommer[2], Y. E. Fillié[1], D. Kornelis[1], R. J. M. van Zeyl[1], G. J. van Dam[1], H. Feldmeier[2] and A. M. Deelder[1] *[1]Laboratory of Parasitology, Medical Faculty, University of Leiden, P.O. Box 9605, 2300 RC Leiden, The Netherlands; [2]Landesinstitut für Tropenmedizin Berlin, Königin-Elisabeth-Strasse 32, 1000 Berlin 19, Federal Republic of Germany*

Abstract

We have developed an enzyme immunoassay (ELISA) for the quantification of the schistosome circulating cathodic antigen (CCA), a glycoprotein associated with the syncitium lining the gut of the parasite. A mouse monoclonal antibody of IgG_3 isotype was used as coating (antigen-capture) antibody, while a biotinylated mouse monoclonal IgM was used as second (antigen-detecting) antibody. Streptavidin-alkaline phosphatase was used as enzyme label. The lower detection limit of the assay was 1·0 ng of the trichloroacetic acid soluble fraction of adult worm antigen (AWA-TCA) per ml, which corresponds to approximately 0·2 ng CCA per ml. The ELISA showed a linear range from 1·0 to 62·5 ng AWA-TCA per ml. Serum and urine samples of 16 individuals infected with *Schistosoma mansoni* (egg counts ranging from 5 to 4820 eggs per gram of faeces) were tested in the assay. Antigen titres ranged from <4–8192. This assay represents a considerable advantage in diagnosis of *Schistosoma* infections as it allows the detection and quantification of CCA in serum and urine in even lightly infected individuals.

Introduction

A variety of antigens secreted or excreted by adult schistosomes is present in the circulation of the infected host. The major circulating antigens belong to the group of gut-associated circulating antigens, consisting of polypeptides and glycoproteins, proteoglycans and polysaccharides. The detection of circulating antigens is a promising method for the diagnosis of human schistosome infections (QIAN & DEELDER, 1982, 1983; ABDEL-HAFEZ *et al.*, 1983; HAYUNGA *et al.*, 1986; FELDMEIER *et al.*, 1986; NOGUEIRA-QUEIROZ *et al.*, 1986; DE JONGE *et al.*, 1988).

Two glycoprotein gut-associated circulating antigens are well described: CAA (circulating anodic antigen) and CCA (circulating cathodic antigen). The presence of CAA in serum or urine reliably indicates an active *Schistosoma* infection, and the effect of chemotherapy can be monitored by assaying CAA in serum or urine (FELDMEIER *et al.*, 1986; DEELDER *et al.*, 1989a, 1989b; DE JONGE *et al.*, 1989a, 1989b, 1989c, in press). However, the low concentration of CAA in the urine requires five-fold concentration of the samples to reach detectable levels with the present assay.

The other major circulating antigen, CCA, also known as antigen M, has been described by CARLIER *et al.* (1975, 1978, 1980a, 1980b), DEELDER *et al.* (1976, 1980), SANTORO *et al.* (1977) and NASH & DEELDER (1985). The polysaccharide nature of this antigen was concluded from its thermostability, solubility in trichloroacetic acid, resistance to protease, ribonuclease, amylase and neuraminidase, and sensitivity to sodium metaperiodate (CARLIER *et al.*, 1978). In contrast to CAA, CCA is positively charged at neutral pH; it is a glycoprotein with a carbohydrate moiety consisting mainly of galactose, fucose, glucosamine and mannose. The antigen shows marked heterogeneity in its molecular weight: CARLIER *et al.* (1978) reported a molecular weight of approximately 45 kDa, while DEELDER *et al.* (1980) reported a range from 50 to over 300 kDa. KESTENS *et al.* (1988) reported the molecular weight of CCA, as derived from sucrose density gradient ultracentrifugation, to be less than 40 kDa.

CCA has been demonstrated in the serum and urine of infected animals and humans and in the mother's milk of infected humans. In hamsters infected with *S. mansoni*, CCA is present in serum and urine within 7 weeks of infection (CARLIER *et al.*, 1978). Using immunofluorescence studies, DEELDER *et al.* (1985) demonstrated CCA from 2 weeks after infection in Kupffer cells, from 3-4 weeks after infection in macrophages in the marginal zones of the spleen, and from 8 weeks after infection in kidney glomeruli, of animals experimentally infected with *S. mansoni*. Specific deposits containing both CCA and CAA have been demonstrated in renal glomeruli of patients with active *S. mansoni* infection (SOBH *et al.*, 1988).

In contrast to CAA, for which assays with a lower detection limit in the nanogram range have been described (DEELDER *et al.*, 1989a; DE JONGE *et al.*, 1989b), no sensitive assay has been described for the quantification of CCA. Although a two-site immunoradiometric assay (NOGUEIRA-QUEIROZ *et al.*, 1986) and an indirect haemagglutination assay (DEELDER *et al.*, 1989b) have been developed, these assays failed to detect antigen in lightly infected individuals. Here we describe a sensitive enzyme-linked immunosorbent assay (ELISA) for the quantification of CCA. Two mouse monoclonal antibodies of different isotype were used: an antibody of immunoglobulin (Ig) G_3 isotype was immobilized on the solid phase, and an IgM coupled to a biotin hydrazide derivative was used as second antibody. Streptavidin/alkaline phosphatase (EC.3.1.3.1) was used as enzyme label, using *p*-nitrophenyl phosphate as the substrate.

Materials and Methods

Sera and standards

Serum and urine samples were collected from individuals from Burundi and Zaire infected with *S.*

*Author for correspondence.

mansoni. All persons gave informed consent. *S. mansoni* infections were assessed by 3 duplicate faecal examinations at one-week intervals using the Kato method (KATZ *et al.*, 1972; POLDERMAN *et al.*, 1985). Serum samples were taken at the third stool examination. All persons were subsequently treated with praziquantel (40 mg/kg orally). Serum samples from 10 healthy Dutch persons, who had never been exposed to *S. mansoni* infection, were used as negative controls. Both positive and negative control samples had been previously included in our studies on circulating anodic antigen detection (DEELDER *et al.*, 1989a; DE JONGE *et al.*, 1989c). All serum samples were treated with trichloroacetic acid before testing to remove interfering proteins and to dissociate immune complexes (DE JONGE *et al.*, 1987). As purified CCA is extremely difficult to obtain in sufficient quantity, the trichloroacetic acid soluble fraction of adult *S. mansoni* worms (AWA-TCA) was used as a standard (DEELDER *et al.*, 1976).

Monoclonal antibodies

Cell line 8-3C10 (IgM isotype) was prepared by fusion of SP2/0 myeloma cells with spleen cells of BALB/c mice immunized with *S. haematobium* vomitus antigen. For cell line 54-5C10-A (IgG_3 isotype), spleen cells of Swiss mice infected with *S. mansoni* (Puerto Rico strain) were used for fusion with SP2/0 mouse myeloma cells. Hybridoma culture supernatants were screened for the presence of antibodies reactive with gut-associated antigens by an immunofluorescence assay on paraffin sections of Rossman's-fixed adult *S. mansoni* worms (NASH, 1978), and by immunoelectrophoresis against the AWA-TCA. Monoclonal antibody 54-5C10-A was purified from ascitic fluid on protein A Sepharose® (Pharmacia Fine Chemicals, Uppsala, Sweden) as described by EY *et al.* (1978). IgM monoclonal antibody 8-3C10 was purified from ascitic fluid by ammonium sulphate precipitation and hydroxylapatite ion exchange chromatography, according to STANKER *et al.* (1985).

Biotinylation of IgM

Monomeric IgM (mouse monoclonal antibody 8-3C10) was biotinylated according to the procedure described by O'SHANNESSY *et al.* (1984, 1987). Three different biotin preparations were used: biotin hydrazide (Pierce, Rockford, USA), biotin aminocaproyl hydrazide (Pierce), and biotin diaminocaproyl hydrazide (Calbiochem, San Diego, USA). Biotin diaminocaproyl hydrazide was selected for subsequent use. The biotinylated IgM was purified using a prepacked, disposable PD-10 column containing Sephadex® G-25 (Pharmacia, Uppsala, Sweden). Glycerol was added to the biotinylated antibody to a final concentration of 50%, and this antibody-conjugate preparation was stored at 4°C until used.

ELISA procedure

After optimization of the assay conditions, the following procedure was adopted. Polyvinylchloride flat-bottom microtitration plates (Flow, Irvine, UK) were coated by adding 100 µl monoclonal antibody solution (5 µg/ml protein A purified antibody in 0·1 M sodium carbonate buffer, pH 9·6) to each well. Plates were incubated for 3 h at 37°C and then washed with 0·002 M phosphate-buffered saline (PBS). The coated wells were blocked by adding 120 µl of bovine serum albumin (BSA; Boehringer, Mannheim, Federal Republic of Germany) solution (250 µg/ml) in 0·1 M sodium carbonate buffer, pH 9·6. After washing, the plates were stored dry at −70°C until used. 80 µl volumes of standards or patients' samples were incubated for one hour at 37°C. 0·035 M PBS, containing 0·02% (v/v) Tween®-20, 0·5% (v/v) polyethylene glycol (PEG)-1000, 0·1% (w/v) BSA, pH 7·8 was used as assay buffer. The plates were then washed, and each well was incubated with 80 µl biotinylated antibody (diluted 1/200 in assay buffer) for one hour at 37°C. After washing, 80 µl streptavidine/alkaline phosphatase (EC. 3.1.3.1) (diluted 1/8000 in assay buffer) solution was added to each well and incubated for 30 min at 37°C. After removing excess conjugate by extensive washing, the amount of coupled conjugate was determined by incubation with 3 mM 1,4-nitrophenyl phosphate (Boehringer, Mannheim, Federal Republic of Germany) in 0·1 M diethanolamine containing 0·5 mM $MgCl_2$, pH 9·6. Substrate incubation was overnight at 4°C. Extinction was read at 405 nm with a Biotek® EL 311 spectrophotometer (Biotek Instruments, Winooski, Vermont, USA). The reciprocal value of the highest serum dilution which showed a colouring above the background level (mean absorbance of buffer controls plus twice the standard deviation) was taken as the end-point titre. A titre <4 was considered as negative, and a titre ≥4 as positive.

Results

Three biotin hydrazide derivatives were used. The best results (i.e. lowest detection limit and highest sensitivity) were obtained with biotin diaminocaproyl hydrazide (Figure).

The sandwich ELISA with purified mouse monoclonal antibody 54-5C10-A (IgG_3) as coating antibody, and mouse monoclonal antibody 8-3C10 (IgM) as biotinylated second antibody, allowed sensitive quantification of CCA. All preparations of monoclonal antibody 8-3C10 conjugated to biotin diaminocaproyl

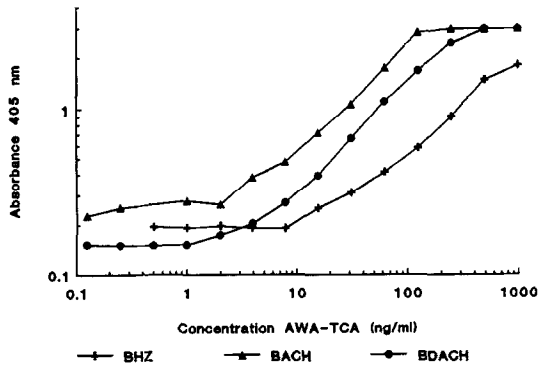

Figure. Dose response curves of monoclonal antibody-based enzyme-linked immunosorbent assay for quantification of the schistosome circulating cathodic antigen using different biotin hydrazide derivates (BHZ=biotin hydrazide, BACH=biotin aminocaproyl hydrazide, BDACH=biotin diaminocaproyl hydrazide); AWA-TCA=trichloroacetic acid soluble fraction of adult worm antigen.

Table. Circulating cathodic antigen titres in serum and urine of individuals infected with *S. mansoni*

Patient no.	Eggs/g	Titre of circulating cathodic antigen	
		Serum	Urine
B.003	660	128	–[a]
B.005	5	<4	–
B.013	928	128	–
B.030	214	16	–
B.031	142	64	–
B.044	5	32	–
B.063	5	<4	–
B.068	130	64	–
B.079	309	128	–
B.089	23	32	–
Z.068	3513	–[a]	8192
Z.155	4820	–	8
Z.312	3873	–	512
Z.398	4320	–	8192
Z.550	2573	–	8192
Z.978	13	–	1024

[a]No specimen available.

hydrazide allowed a lower detection limit of 1·0 ng AWA-TCA per ml, determined by calculating the mean plus twice the standard deviation of 12 replicate measurements of buffer sample. Standard curves were obtained by assaying AWA-TCA serially diluted in assay buffer. The CCA content of this preparation was estimated to be around 20% on the basis of gel-filtration and ion-exchange chromatographic purification studies (data not shown). The measuring range of the ELISA was 1·0–250 ng AWA-TCA per ml with a linear dose response curve from 1·0 to 62·5 ng/ml (Figure).

Neither serum nor urine samples of negative controls gave any false positive result, suggesting a very high specificity. CCA was assayed in serum samples from 10 patients from Burundi and 6 urine samples of patients from Zaire. Counts of eggs per gram (epg) and CCA titres in serum and urine are given in the Table. The intra-assay imprecision of the test was 7·0–9·6%, the inter-assay variation was 7·7–11·5%. The analytical recovery of positive samples ranged from 92·5–98·1%.

Discussion

In searching for a sensitive assay for the quantification of the schistosome CCA we applied a biotinylation procedure (O'SHANNESSY *et al.*, 1984, 1987) to a number of mouse monoclonal antibodies raised against the antigen. Based on their reactivity in the immunofluorescence assay, their specificity for CCA shown by immunoelectrophoresis, and their reactivity in an ELISA, several monoclonal antibodies of different isotypes (IgG_1, IgG_3 and IgM) were selected from a large panel of monoclonal antibodies for further study of their applicability in the ELISA. The best results were obtained by using monoclonal antibody from cell line 54-5C10-A (of IgG_3 isotype) as coating antibody, and monoclonal antibody from cell line 8-3C10 (of IgM isotype) as biotinylated second antibody.

In the earlier study, using an indirect haemagglutination assay with a lower detection limit of 50 ng AWA-TCA per ml (DEELDER *et al.*, 1989b), we were not able to detect antigen in patients excreting fewer than about 500 epg. NOGUEIRA-QUEIROZ *et al.* (1986), using a two-site immunoradiometric assay, reported a lower detection limit of 5 ng of circulating schistosome antigen in blood and in urine from patients with schistosomiasis, although FELDMEIER *et al.* (1986), using the monoclonal antibody in the same assay, reported a lower detection limit of 150 ng of antigen per ml. A strong correlation was reported between the egg excretion rate in stool and the concentration of CCA in serum of patients with *S. mansoni* infection. However, in patients with mixed *S. haematobium* and *S. mansoni* infections, no significant relationship could be found between CCA levels and egg excretion in urine. The detectability of the ELISA described here (lower detection limit 1·0 ng AWA-TCA per ml) allows a very sensitive quantification of CCA in patients' samples.

We previously demonstrated the presence of CAA and CCA in urine of patients with *S. mansoni* and *S. haematobium* infections (DE JONGE *et al.*, 1989c). Antigen could be demonstrated in most of the patients, and a good correlation was found between antigen titres in serum compared with those in urine and between antigen titres (in either serum or urine) and egg excretion in *S. mansoni* infections. In that preliminary study, using an indirect haemagglutination assay for the quantification of CCA and an ELISA for the quantification of CAA, we found high levels of CCA in urine of schistosomiasis patients (data not shown). This is important because it would allow the determination of antigen in urine without the need for concentration of the samples, as is the case for the quantification of CAA in urine. With the sensitive assay for the quantification of CCA described here, a non-invasive immunodiagnosis of *Schistosoma* infections is within sight.

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