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Immunoblot analysis of *Schistosoma mansoni* antigens with sera of schistosomiasis patients: diagnostic potential of an adult schistosome polypeptide

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SUMMARY

We compared the reaction in immunoblots of sera obtained from patients with parasitologically proven *S. mansoni* infections, with a suspected history of schistosomiasis infection, or with unrelated parasitic diseases. Several polypeptides from adult *S. mansoni* reacted with the schistosomiasis patients' sera in a heterogeneous manner. However, a component of approximately 31 kilo daltons (kD) reacted with all schistosomiasis sera and with several sera of suspected schistosomiasis cases. No reaction was ever observed with sera of patients harbouring other parasites. Thus, the polypeptide has potential diagnostic value. The use of sera of patients with recent infections demonstrated that: (a) the earliest time of antibody formation against the 31 kD component was approximately 40 days post infection, (b) the reaction with this polypeptide in immunoblots was exceptionally strong and (c) antibodies directed against other schistosome proteins were barely detectable at this time. Identical results were obtained with sera of experimentally infected mice. The 31 kD component was present in parasites of either sex. It was apparently not a glycoprotein. Evidence suggests that the 31 kD polypeptide may originate from the schistosome gut.

Keywords immunoblots immunodiagnosis *Schistosoma mansoni* schistosome antigen

INTRODUCTION

Immunodiagnosis of schistosomiasis has for some time been performed using whole or sectioned parasites as well as crude or partially purified parasite extracts (Kagan & Pellegrino, 1961; Kagan, 1968; Mott & Dixon, 1982). A number of more purified schistosome-specific antigens have been characterized (Pelley, Warren & Jordan, 1977; Kelsoe & Weller, 1978; Nash, 1978; Rotmans, 1978; Senft *et al.*, 1979; Deelder *et al.*, 1980; Nash, Lunde & Cheever, 1981; Dunne *et al.*, 1984; Norden & Strand, 1984). Their application in diagnostic tests may be complicated by the relatively sophisticated techniques required for their preparation and/or by the carbohydrate nature that excludes their production by recombinant DNA technology. Recent evidence (Ruppel *et al.*, 1985) demonstrated that total adult worm proteins separated by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) contain a number of components reacting in immunoblots with sera from *S. mansoni*-infected mice. The technique is simple in that it does not involve any fractionation of schistosomes prior to PAGE. It demonstrates the consistent

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recognition of a 31 kD component by all sera of individual mice harbouring a variable number of worms for a variable duration of infection. Here we investigate the reaction of schistosome proteins with antibodies in sera obtained from schistosomiasis patients and explore the diagnostic value of a 31 kD schistosome component.

MATERIALS AND METHODS

Parasites. Schistosomes of a Puerto-Rican strain (Ruppel, Rother & Diesfeld, 1982) were obtained from NMRI-mice 6–7 weeks following percutaneous infection (Smithers & Terry, 1965) with 200–300 cercariae. Perfusion of the mice was done with warm Dulbecco's Modified Eagle's medium (DME, Gibco-Europe, Karlsruhe, FRG, no. 074-1600) supplemented with 5% inactivated newborn calf serum (NCS, Gibco) and 10 Units of heparin per ml. The worms were washed twice in DME-NCS and twice in DME. Schistosomes to be used for electrophoresis were stored in a minimum amount of DME at −40°C. Worms to be used in immunofluorescence were immersed in tissue teck II O.C.T. (Miles, Munich, FRG) prior to freezing.

Electrophoresis and immunoblotting. Schistosomes were suspended in sample buffer (0·5 M Tris-HCl pH 6·8, 10% glycerol, 5% 2-mercaptoethanol, 3% sodium dodecylsulfate and traces of bromophenolblue), sonicated and boiled for 2 min. Eight pairs in about 1 ml of sample buffer were applied onto 2 mm thick 10 by 9 cm slab gels without wells in the spacergel. Gels were prepared as peviously described (Laemmli, 1970; Ruppel & Cioli, 1977). Electrophoretic transfer (Towbin, Staehlin & Gordon, 1979) of proteins to nitrocellulose (Schleicher & Schüll, Dassel, FRG; BA85, pore size 0·45 μm) was done at 6 V/cm and 250 mA overnight at 4°C in 0·025 M Tris, 0·168 M glycine and 25% methanol. The nitrocellulose sheet was cut into 30–40 strips. Immune recognition was performed with individual sera diluted 1:150 unless stated otherwise. Previously described washing and incubation conditions (Lucius, Rauterberg & Diesfeld, 1983) were followed except that 96% pure bovine serum albumin (Serva, Heidelberg, FRG) was used to saturate the protein binding sites of the nitrocellulose. Immune complexes were detected with a 1:500 dilution of horseradish peroxidase-conjugated goat antibodies to human IgG (gamma chain specific, affinity purified, Tago, Burlingame, CA, USA) followed by incubation of the nitrocellulose in 6% of a stock solution of 4-chloro-1-naphthol (Merck, Darmstadt, FRG; 0·3% 4-chloro-1-naphthol in methanol) and 0·02% H_2O_2 in water (Hawkes, Niday & Gordon, 1982). The staining reaction was stopped by washing when a background staining began to appear. The total pattern of proteins transferred to the nitrocellulose was revealed by incubating single strips after the transfer of proteins in 0·2% amidoblack (Amidoschwarz, Merck, Darmstadt, FRG) in 40% methanol and 7% acetic acid. Blots were photographed on Agfa Ortho 25 film.

Sera. Sera were obtained predominantly from German donors at the Institute for Tropical Hygiene unless stated otherwise. Patients excreting *S. mansoni* eggs had either travelled through, or lived for longer periods in, endemic areas. The maximum duration of infection ranged from less than 2 months to 13 years after the first possible contact with infected water. All of these patients had schistosome-specific antibodies demonstrable either in the immunofluorescence assay (IFA) described below, in an enzyme-linked immunosorbent assay (ELISA) described previously (Ruppel *et al.*, 1983) or in both tests.

For patients numbers 12, 15, 16 and 17, a datable period of 6–10 days for contact with infected water could be calculated from travel routes through Africa, bathing occasions and endemicity of schistosomiasis. The middle of this period was set at as date of infection. The first serum samples of these patients were obtained 28–44 days after infection. At this time schistosome eggs were undetectable in the faeces by the merthiolate-iodine formalin concentration technique. In patients 12, 15 and 16, *S. mansoni* eggs were first detected on days 48, 42 and 49, while *S. haematobium* eggs were not detected in the urine, collected over 24 h, on days 47, 41 and 48, respectively. Patient 17 had travelled together with patients 15 and 16, developed the same symptoms of disease and had similar antibody titres in the IFA. For these reasons the patient was included in this study, although *S. mansoni* eggs were not detected in his faeces, probably due to praziquantel chemotherapy on day 43 after infection.

In a group of additional patients, active or past schistosome infections were suspected, although parasite eggs were not detectable.

Patients infected with *Entamoeba histolytica* exhibited positive reactions in each of three tests (IFA, counter immunoelectrophoresis and indirect haemagglutination). Malaria patients infected with *Plasmodium falciparum, P. malariae* or *P. vivax* were identified either parasitologically by IFA or by both techniques. Patients infected with *Toxoplasma gondii* had specific IFA titres of over 1:4,000. Infections with *Echinococcus* spec. were confirmed by surgery or positive reactions in each of three diagnostic tests (IFA, indirect haemagglutination, counter immunoelectrophoresis). Kala-Azar patients were from Kenya; infections with *Leishmania donovani* were diagnosed by spleen biopsies and an ELISA (Jahn & Diesfeld, 1982). Patients infected with *Opistorchis viverrini* were from Thailand and excreted eggs. Patients infected with *Onchocerca volvulus* were from West Africa and had microfilariae in skin snips. In many of the patients, parastic infections in addition to those specifically quoted were diagnosed. Control serum was obtained from German individuals without any known history of current or past parasite infections.

Immunofluorescence assay. Cryostat sections (8 μm) of schistosomes were fixed in methanol for 10 min at 4°C. Incubations with serum diluted in phosphate buffered saline (PBS) were for 30 min at 37°C. Following three washes in PBS, the sections were incubated as above with a 1:150 dilution of FITC-anti-human IgG (Behringwerke, Marburg, FRG) in 0·01% (w/v) Evans blue (Merck, Darmstadt, FRG). Following three washes, the sections were covered with 10% glycerol in PBS. Fluorescence was photographed on Kodak Ektachrome film.

RESULTS

Separation of complete adult schistosomes by SDS-PAGE resulted in a complex pattern of protein bands in accordance with earlier results (Ruppel & Cioli, 1977). Several of the components reacted in immunoblots with sera obtained from egg-excreting *S. mansoni* patients (Fig. 1A). The reacting polypeptides represented minor components as detected by staining for total schistosome proteins. In fact, a predominant band in immunoblots corresponded to a relatively faint band in the

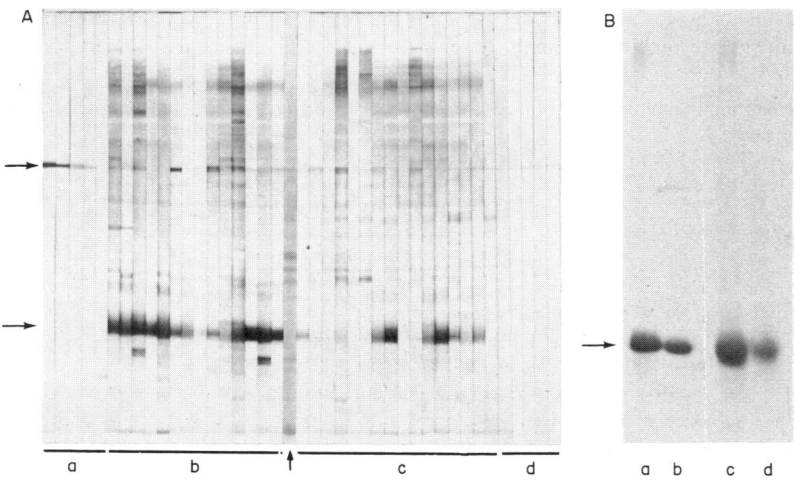

Fig. 1. Immunoblot analysis of adult schistosome antigens recognized by human sera.

(A) Reactions with sera obtained from: (a) five uninfected persons; (b) patients 1–14 excreting *S. mansoni* eggs; (c) 16 patients with suspected *S. mansoni* infection; (d)five echinococcosis patients.

(B) Reaction of serum from patient 12 (44 days after infection diluted 1:300) with (a, c) female or (b, d) male schistosome proteins following PAGE under (a, b) reducing or (c, d) non reducing conditions.
Gels contained 10% acrylamide.

The positions of polypeptides with 31 kD and 67 kD are indicated by, respectively, upper and lower arrows. Bottom arrow indicates lane stained for total protein.

amidoblack staining. The apparent molecular weight of this polypeptide was 31 kD. It reacted with sera of all of the proven and several of the suspected schistosomiasis patients. Sera of control persons without any diagnosed or suspected parasitic infection did not react with the 31 kD polypeptide (Fig. 1A, Table 1). A 68 kD component was also recognized by a number of sera from schistosomiasis patients, but a few persons lacking any previous contact with schistosomes also had antibodies to the 68 kD protein. The reactions with several other schistosome components were either faint or observed only with part of the patient sera. Thus the 31 kD protein is the only antigen being consistently and, in most cases, predominantly recognized by sera from *S. mansoni* patients.

Sera obtained from patients with unrelated parasitic infections were examined for crossreactivity with schistosome proteins. At most, sporadic reactions with a few proteins occurred as illustrated for echinococcosis patients in Fig. 1A. No reaction with the 31 kD component was observed with any serum obtained from 97 patients with unrelated parasitic diseases (Table 1). Within the range of parasitic diseases tested, the 31 kD schistosome polypeptide was, therefore, specifically recognized by sera from schistosomiasis patients.

Antibodies against the 31 kD polypeptide were present as early as 44 days following infection in patient number 12. The antibody response of this patient, shown in Fig. 2, was at all points of time directed predominantly against the 31 kD component. This was confirmed with three additional patients (15, 16 & 17) of a travel group who presented with an equally well defined duration of infection. All of them developed a strong antibody response that was nearly exclusively directed against the 31 kD component (Fig. 2). In particular in patients 15 and 16 a rapid conversion from unresponsiveness to strong responsiveness against the 31 kD polypeptide was observed in the presence of an unchanged background reactivity with a few other components. Antibodies against the 31 kD protein were detected as early as 35–49 days post infection, i.e. at a time when schistosome eggs were not yet detectable in the faeces of all of the patients.

The findings were reproduced in experimental infections of mice. Thus, sera of mice obtained 28 days after infection with 1,000 cercariae reacted strongly with a 31 kD component in immunoblots (Fig. 2). Mice infected for the same time with 300, but not those infected with 50 cercariae were previously shown to form antibodies against the 31 kD polypeptide 28 days after infection (Ruppel *et al.*, 1985).

Some of the properties of the 31 kD polypeptide were studied. It was present in both male and female schistosomes (Fig. 1B). The 31 kD polypeptide was observed following electrophoretic separation of schistosome components under reducing (in the presence of 2-mercaptoethanol) or

Table 1. Recognition in immunoblots of a 31 kD schistosome polypeptide by sera from patients infected with different parasites

	Reaction*	
Information	Positive	Negative
Schistosoma mansoni	21	0
Entamoeba histolytica	0	14
Toxoplasma gondii	0	8
Leishmania donovani	0	19
Plasmodium spec.	0	25
Opistorchis viverrini	0	17
Echinococcus spec.	0	8
Onchocerca volvulus	0	6
None	0	24
Total *Schistosoma mansoni*	21	0
Total others	0	121

* Number of sera tested is number of positive plus negative reactions.

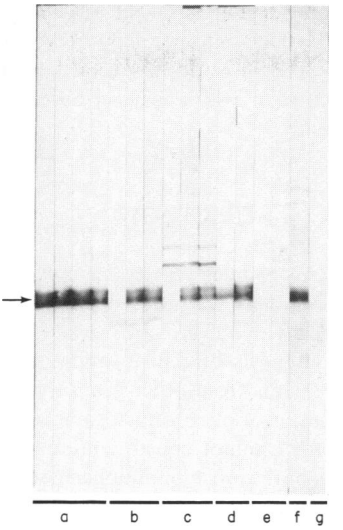

Fig. 2. Reactions with adult schistosome antigens of sera obtained early after infection with *S. mansoni*. Immunoblot with sera from: (a) patient 12 on days 44, 48, 52 and 58, (b) patient 15 on days 28, 42 and 81, (c) patient 16 on days 35, 49 and 70 and (d) patient 17 on days 35 and 61 after infection, (e) sera of two control persons, (f) pool of sera from 5 NMRI-mice infected with 1000 cercariae for 28 days and (g) serum of an untreated mouse. The gel contained a 10–14% acrylamide gradient. The arrow indicates the position of the 31 kD polypeptide.

non reducing (without 2-mercaptoethanol) conditions (Fig. 1B). This suggests that the native protein is not an oligomer. It was not detectable by carbohydrate staining and thus is probably not a glycoprotein (data not shown).

The possible morphological localization of the 31 kD polypeptide was investigated by immunofluorescence in sectioned schistosomes. All schistosomiasis patients of this study had antibodies reacting with the schistosome gut, including patients 12 at a time before eggs were detected in the stools (Fig. 3). When the immunoblot staining intensity of the 31 kD protein was

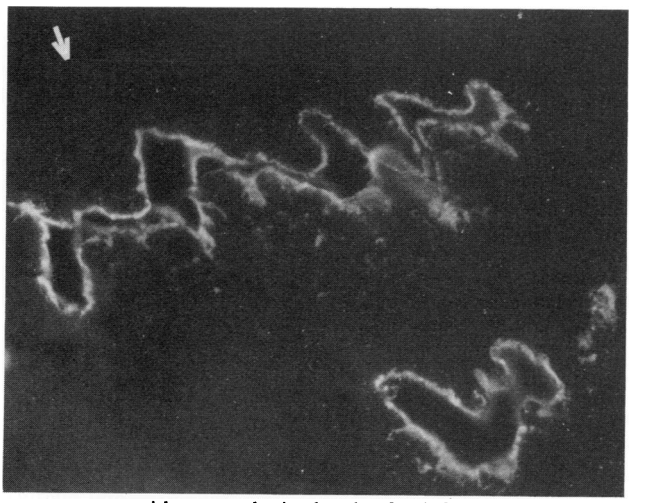

Fig. 3. Immunofluorescence assay with serum obtained early after infection. IFA was performed with a 1:640 dilution of serum from patient 12 obtained 44 days after infection. Fluorescence is present in the schistosome gut and absent from the tegument (arrow).

plotted for individual sera against the IFA-titres of the gut fluorescence, a linear dependency was observed (correlation coefficient $r^2 = 0{\cdot}65$; data not shown). Variable but weaker responses to the parenchyma and parasite surface were observed in several schistosomiasis patients. Schistosome gut associated fluorescence with a titre of > 40 was never observed using sera of patients suffering exclusively from other parasitoses.

DISCUSSION

A 31 kD polypeptide of adult schistosomes reacted in immunoblots with all sera of patients excreting *S. mansoni* eggs. The component was not recognized by sera of patients with unrelated parasitic diseases. The data obtained with our presently available sera suggest the usefulness of the 31 kD polypeptide for immunodiagnosis of schistosomiasis mansoni. Further studies should substantiate this contention for patients from other geographic areas, for those with very long-standing infections and with infections of other schistosome species.

The 31 kD polypeptide was only a minor constituent among the total schistosome proteins. Nevertheless, it appeared to be one of the most immunogenic schistosome polypeptides detected in our assay. Indeed, it was the single most important component reacting with sera obtained early after infection from humans and mice. Moreover, it was one of the components giving the strongest reactions with sera obtained in later infections (Fig. 1; Ruppel *et al.*, 1985). The strong immunogenicity of the 31 kD polypeptide may facilitate detection of the corresponding antibodies in an immunodiagnostic test. Since the component is protein in nature and contains at most few carbohydrates, it may eventually be produced in large quantities by recombinant DNA technology.

The parasite developmental stages inducing the antibody response against the 31 kD polypeptide most likely are the developing or adult worms. A role of cercariae and very young schistosomula appears unlikely since mice vaccinated with lethally irradiated cercariae failed to produce detectable antibodies against the component (Ruppel *et al.*, 1985). A role for eggs was excluded here by the use of early infection sera and of male schistosomes as antigen source.

The immune response against schistosome-specific proteins has been the subject of several recent publications. Sera from *S. mansoni* infected patients immunoprecipitated more than nine *S. mansoni*-glycoproteins (Norden & Strand, 1984). Two of these glycoproteins with a molecular weight of 40 and 70 kD, respectively, were specific for the genus *Schistosoma*, whereas glycoproteins in the 31 kD range were absent or present only as a minor component. Since proteins other than glycoproteins were excluded and since the immunorecognition step was performed under non denaturing conditions, the presently described 31 kD component probably was not detected. Schistosome malate dehydrogenase was the predominant component reacting in immunoelectrophoresis with sera of infected mice (Rotmans, 1978). The enzyme has a molecular weight of more than 60 kD and reacted with only a few sera of schistosomiasis patients. It must be different from our 31 kD polypeptide, of which oligomers were not observed.

Potentially immunodiagnostic antigens were detected in *S. mansoni* eggs (Pelley *et al.*, 1977; Dunne *et al.*, 1984) and among in-vitro translation products from poly $(A)^+$ mRNA obtained from *S. mansoni* eggs, but not adult parasites (Cordingley *et al.*, 1983). All egg antigens appear, however, unrelated to our 31 kD component, which was obtained from adult parasites of both sexes and recognized by antibodies formed prior to the beginning of egg excretion by the patients.

The correlation between a strong reaction with the 31 kD polypeptide in immunoblots and a high antibody titre in the IFA against the gut agrees with our results in murine schistosomiasis. The two reactions were concomitantly observed following infections with normal cercariae and were both absent following vaccination with lethally irradiated cercariae (Ruppel *et al.*, 1985). The data suggest that the schistosome intestine may be the origin of the 31 kD protein. The data obviously do not exclude the possibility that additional gut associated antigens may be detected in the IFA.

Several gut associated antigens have been described. The 31 kD polypeptide very likely is different from gut antigens of a predominantly carbohydrate nature and relatively high molecular weight (Kelsoe & Weller, 1978; Nash, 1978; Deelder *et al.*, 1980; Nash *et al.*, 1981; Carlier *et al.*, 1980). A haemoglobinolytic enzyme in the schistosome gut (Timms & Bueding, 1959) was shown to

have a molecular weight ranging between approximately 27 kD (Sauer & Senft, 1972) and 32 kD (Deelder, Reinders & Rotmans, 1977). Infected humans and experimental animals including mice were shown to form homocytotropic antibodies against this enzyme (Senft & Maddison, 1975; Deelder *et al.*, 1977) which were strongly schistosome species-specific (Senft *et al.*, 1979). A haemoglobinolytic enzyme was also detected in the serum of infected mice (Senft, Goldberg & Byram, 1981). Available data does not permit a conclusion as to whether the protein(s) investigated by Senft, Deelder and coworkers, and by ourselves, are identical, although molecular weights and localization in the gut are similar.

In conclusion, the presently described techniques permit for the first time the easy detection of IgG-antibodies directed predominantly and specifically against one polypeptide antigen of *S. mansoni* in the sera of infected humans.

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