Periodate-sensitive immunological cross-reactivity between keyhole limpet haemocyanin (KLH) and serodiagnostic *Schistosoma mansoni* egg antigens

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SUMMARY

Both CEF6, a cation-exchange fraction of soluble *Schistosoma mansoni* egg antigens (SEA), composed of the 2 antigens, alpha-1 and omega-1, and haemocyanin from the keyhole limpet, *Megathura crenulata*, have shown potential for immunodiagnosis of human schistosomiasis. Possible cross-reactivity between antigens in SEA and keyhole limpet haemocyanin (KLH) was explored by Western immunoblotting and enzyme-linked immunosorbent assay (ELISA) using sera from rabbits immunized with KLH, SEA, CEF6, alpha-1, omega-1, or egg antigen k5. Both immunoassays revealed a high degree of serological cross-reactivity between the schistosome egg antigens and KLH, much of it due to sodium periodate-sensitive epitopes. Cross-reactivity with schistosome antigens with proven diagnostic efficacy may thus, in part, explain the usefulness of KLH for the diagnosis of human schistosomiasis mansoni.

Key words: serodiagnosis, keyhole limpet haemocyanin (KLH), CEF6, cross-reactivity.

INTRODUCTION

The demonstration of ova in the faeces or urine and egg morphology have been the traditional bases for the diagnosis of schistosomiasis. A number of techniques have been developed for detecting and quantifying ova but egg detection lacks sensitivity especially with respect to lightly infected individuals who may have very low egg output. Undetected infections may be important reservoirs in the persistence of transmission following control programmes (Gryseels *et al*. 1991, 1994; Butterworth *et al*. 1991) and this may be particularly important post-chemotherapy when egg counts fall, leading to an overestimation of cure rates (da Cunha, Cancado & de Rezende, 1987). Serodiagnostic tests have thus been developed to improve the sensitivity and specificity of diagnosis of schistosomiasis.

In the collaborative study on antigens for the immunodiagnosis of schistosomiasis organized by The World Health Organization in 1981, *S. mansoni* egg antigens demonstrated a higher sensitivity and specificity in ELISA than adult worm antigens for the diagnosis of active *S. mansoni* infections before and after specific treatment (Mott & Dixon, 1982). Serology to *S. mansoni* SEA also correlates fairly well with faecal egg densities as demonstrated in Ethiopian schoolchildren (Eltiro, Ye-Ebiyo & Taylor, 1992).

Antibody activity against keyhole limpet haemocyanin has been used to detect *S. mansoni* infection in Brazil (Alves-Brito *et al*. 1992), *S. mansoni* and *S. haematobium* infections in Egypt (Markl *et al*. 1991) and *S. japonicum* infections in China (Yuesheng *et al*. 1994). KLH has also been used to differentiate between acute and chronic schistosomiasis mansoni (Mansour *et al*. 1989), acute and chronic *S. japonicum* infections (Yuesheng *et al*. 1994) and for the diagnosis of acute *S. mansoni* (Alves-Brito *et al*. 1992).

Here we present evidence of the existence of considerable immunological cross-reactivity between KLH and *S. mansoni* egg antigens. The importance of carbohydrate epitopes in this cross-reactivity is demonstrated and we suggest that the presence of shared carbohydrate epitopes in KLH and SEA may, in part, explain the usefulness of KLH as a serodiagnostic agent for schistosomiasis.

MATERIALS AND METHODS

Antigen preparation

Soluble egg antigen (SEA) was prepared from *S. mansoni* eggs recovered from livers and intestines of male random-bred T. O. strain mice infected with a Puerto Rican strain of *Schistosoma mansoni* as previously described (Doenhoff *et al*. 1981). Com-

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mercially available KLH was used in this study (Sigma, Poole, UK).

Sodium periodate treatment of antigens

S. mansoni SEA and KLH were exposed to varying concentrations (0–50 mM) of sodium periodate in 0·1 M sodium acetate buffer (pH 4·5) overnight in the dark at 4 °C. The reaction was stopped by incubation with approximately equal volumes of 50 mM sodium borohydride for 30 min at room temperature. The treated antigen was analysed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) under non-reducing conditions. Coomassie blue-stained SDS–PAGE indicated that proteins in SEA were degraded at sodium periodate concentrations greater than 20 mM as bands were lost or disrupted (results not shown). At 20 mM no such degradation of the proteins was apparent and this concentration was used for subsequent immunoblotting and ELISA experiments.

Rabbit antisera

Rabbit antisera against unfractionated *S. mansoni* SEA, *S. mansoni* egg antigens alpha-1, omega-1 and k5, and *S. mansoni* egg antigen fraction CEF6 were produced using previously described methods (Dunne *et al*. 1986). CEF6 was purified from SEA by cation exchange chromatography using a Sephadex CM-50 (Pharmacia) cation-exchange column (McLaren *et al*. 1981). Commercially available anti-KLH serum was used (Sigma, Poole, UK).

SDS–PAGE and Western blotting

Discontinuous SDS–PAGE, adapted from previously published methods (Laemmli, 1970) was performed under non-reducing conditions using a 3% stacking gel and a 12% resolving gel for *S. mansoni* SEA and an 8% resolving gel for KLH. A mixture of 25 μg SEA or KLH in 100 μl of distilled water plus 25 μl of sample buffer (2·5 ml glycerol, 0·10 g sodium dodecyl sulfate, 2·25 ml of 0·315 M Tris–HCl) was loaded per gel. Low-molecular weight markers (Pharmacia, LKB Biotechnology, UK) were simultaneously electrophoresed. Proteins were transferred onto nitrocellulose paper (Hybond-C Super, Amersham, UK) following electrophoresis according to previously published methods (Towbin, Staehelin & Gordon, 1979).

Periodate treatment was performed according to previously published methods (Woodward, Young & Bloodgood, 1985). The blots were equilibrated for 30 min at room temperature in 0·1 M sodium acetate buffer (pH 4·5), then cut in half and incubated in either 20 mM sodium periodate in 0·1 M sodium acetate buffer or in 0·1 M sodium acetate buffer alone, overnight in the dark at 4 °C. Following extensive washing in 0·1 M sodium acetate buffer the blots were incubated in 50 mM sodium borohydride in phosphate-buffered saline (PBS, 145 mM sodium chloride, 2 mM sodium dihydrogen orthophosphate, 4 mM disodium hydrogen orthophosphate, pH 7·2) for 30 min and subsequently washed extensively in Tween transblotting solution (TTBS, 20 mM Tris, 0·9% NaCl, 0·1% Tween 20, pH 7·2) and blocked for 2 h in a 1% non-fat milk solution. The blots were then cut into strips and probed with rabbit antisera (1:200 dilution) raised against *S. mansoni* egg antigens and KLH. Horse-radish peroxidase-labelled goat anti-rabbit IgG (1:1000 dilution, Nordic Immunoconjugates) was used as secondary antibody. The blots were developed by the addition of 4, chloro-1-naphthol (20 mg in 4 ml of methanol) in 16 ml of trans-blotting solution (TBS, 20 mM Tris, 0·9% NaCl, pH 7·2) containing hydrogen peroxide (10 μl of 30% v/v H_2O_2). The reaction was stopped by immersing the blots in distilled water.

Enzyme-linked immunosorbent assay

Microtitre plates (Type M29A, F-Form, PS Microplates, Dynatech, West Sussex, UK) were coated with either 2 μg SEA or KLH per plate in carbonate buffer (15 mM sodium carbonate, 35 mM sodium hydrogen carbonate, pH 9·6), overnight at 4 °C. Coating conditions were determined by checkerboard titration. One hundred microlitre volumes per well of antigen solution, primary and secondary antibodies and substrate were used throughout.

Periodate treatment of antigens in ELISA was modified from Dunne *et al*. 1988*b*). Following 3 washes in PBS + Tween-20 (145 mM sodium chloride, 2 mM sodium dihydrogen orthophosphate, 4 mM disodium hydrogen phosphate, 0·05% Tween 20, pH 7·2) the plates were incubated with 0·1 M sodium acetate buffer for 30 min at room temperature and washed 3 times with the same solution. One half of the plate was incubated with 20 mM sodium periodate in 0·1 M sodium acetate buffer, the other half was incubated with 0·1 M sodium acetate buffer alone overnight, in the dark, at 4 °C. After treatment the plates were incubated with 50 mM sodium borohydride in PBS for 30 min then washed 3 times with PBS + Tween-20. The plates were blocked with 1% bovine serum albumin for 2 h at room temperature. Following 3 washes in PBS–Tween the plates were incubated with antisera raised against SEA and KLH (1:200 dilution) for 2 h at room temperature. Horse-radish peroxidase-labelled, goat anti-rabbit IgG conjugate, (1:1000 dilution) was used as secondary antibody. The extent of binding was measured colorimetrically after addition of 300 μl of 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (300 μl of a 2 mg/ml solution diluted in 9·7 ml of 2·3% citric acid solution, pH 4·0) in the presence of hydrogen peroxide (10 μl of 30% v/v H_2O_2).

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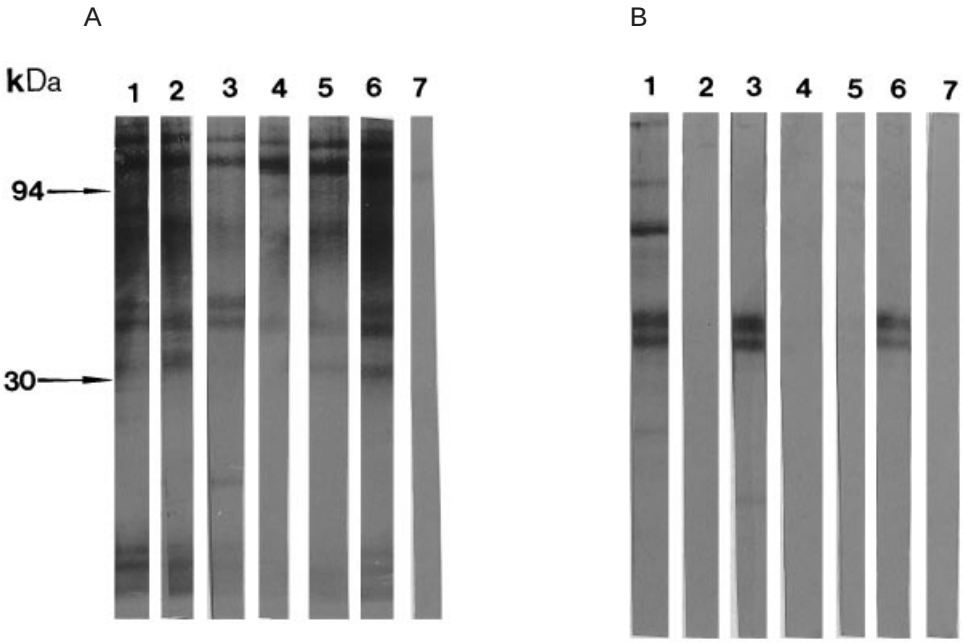

Fig. 1. Reactivity of rabbit antisera raised against *Schistosoma mansoni* egg antigens or KLH against *S. mansoni* egg antigens. (A) Western blot of *S. mansoni* SEA treated with 0·1 M sodium acetate buffer alone and probed with Lane 1, anti-SEA; Lane 2, anti-KLH; Lane 3, anti-alpha-1; Lane 4, anti-omega-1; Lane 5, anti-k5; Lane 6, anti-CEF6; Lane 7, control serum. (B) Effect of periodate treatment. Western blot of *S. mansoni* SEA treated with 20 mM sodium periodate in 0·1 M sodium acetate buffer alone and probed with Lane 1, anti-SEA; Lane 2, anti-KLH; Lane 3, anti-alpha-1; Lane 4, anti-omega-1; Lane 5, anti-k5; Lane 6, anti-CEF6; Lane 7, control serum.

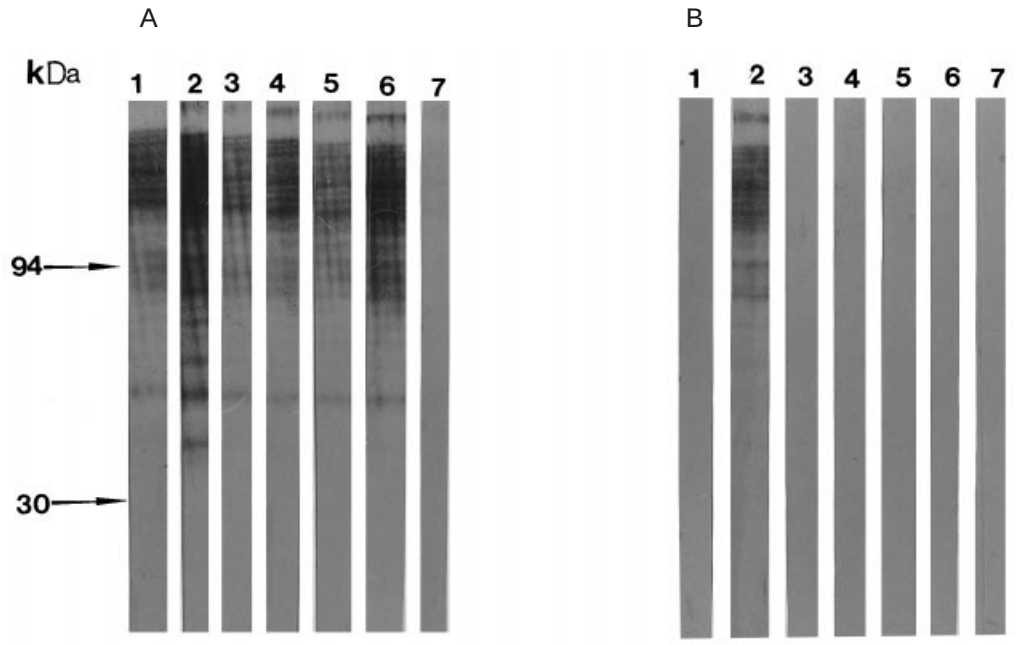

Fig. 2. Reactivity of rabbit sera raised against *Schistosoma mansoni* egg antigens or KLH against KLH. (A) Western blot of KLH treated with 0·1 M sodium acetate buffer alone and probed with Lane 1, anti-SEA; Lane 2, anti-KLH; Lane 3, anti-alpha-1; Lane 4, anti-omega-1; Lane 5, anti-k5; Lane 6, anti-CEF6; Lane 7, control serum. (B) Effect of periodate treatment. Western blot of KLH treated with 20 mM sodium periodate in 0·1 M sodium acetate buffer alone and probed with Lane 1, anti-SEA; Lane 2, anti-KLH; lane 3, anti-alpha-1; Lane 4, anti-omega-1; Lane 5, anti-k5; Lane 6, anti-CEF6; Lane 7, control serum.

RESULTS

Fig. 1A shows that a rabbit antiserum raised against KLH gave considerable immunological cross-reactivity with antigens present in *S. mansoni* SEA. The pattern of reactivity of the anti-KLH serum (Lane 2, Fig. 1A) is quite similar to that of the anti-whole SEA and anti-CEF6 sera (Lanes 1 and 6 respectively, Fig. 1A). Egg antigens that the anti-KLH serum was particularly reactive with included

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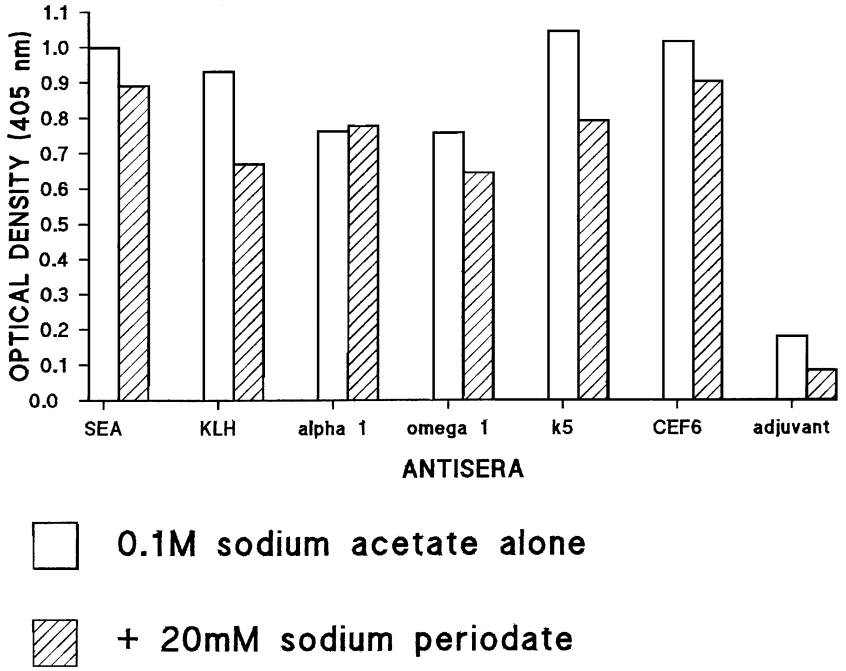

Fig. 3. Reactivity of rabbit sera raised against *Schistosoma mansoni* egg antigens or KLH against untreated or periodate-treated SEA in ELISA.

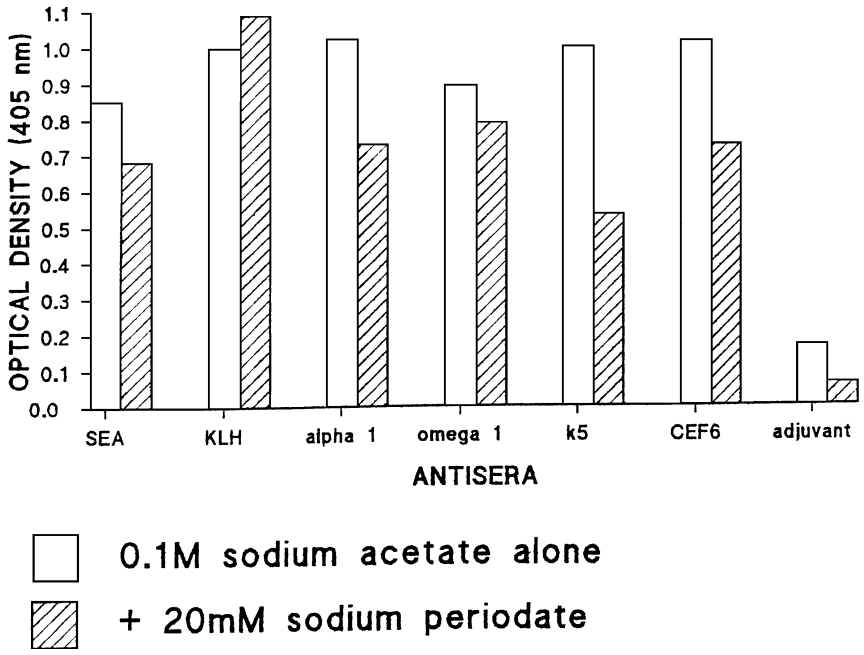

Fig. 4. Reactivity of rabbit antisera raised against *Schistosoma mansoni* egg antigens or KLH against untreated or periodate-treated KLH in ELISA.

one with a molecular weight of > 94 kDa and a doublet consisting of an upper band at 41 kDa and a lower 36 kDa band, a 31 kDa antigen and a 65 kDa antigen. Comparison with the more specific sera that had been raised against individual egg antigens indicates that the > 94 kDa antigen is likely to be k5 (Fig. 1A, lane 5) and the 36/41 kDa doublet is alpha-1 (Fig. 1A, lane 3). The cross-reactivity of unabsorbed rabbit antisera raised against egg antigens alpha-1 and omega-1 with the k5 antigen has been noted previously (Dunne *et al*. 1986).

Conversely, antisera raised against SEA, CEF6, alpha-1, omega-1 and k5 demonstrate a blotting pattern against KLH which was very similar to that produced by the anti-KLH serum (Fig. 2A). The egg antisera appear to respond to a number of antigens in KLH ranging from 50 to > 94 kDa.

The ELISA data also demonstrated that anti-KLH antibodies are highly cross-reactive with SEA (Fig. 3) and that the anti-egg sera tested cross-react with KLH (Fig. 4).

Periodate treatment of the antigens used in

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Western blotting effected a substantial reduction in the capacity of the anti-KLH serum to recognize *S. mansoni* egg antigens (Fig. 1B, lane 2a). Similarly, periodate treatment of KLH obliterated the reactivity of this antigen with anti-egg antibodies (Fig. 2B). Figure 1B shows that the antigenicity of the 36/41 kDa doublet (alpha-1) and the 65 kDa egg antigen survived periodate treatment. In fact, the reactivity of the anti-alpha-1 antiserum was seemingly enhanced and rendered more specific by treatment of the antigen with periodate. Disruption of the carbohydrate epitopes of the SEA used for ELISA also resulted in a decrease in recognition of SEA antigens by the anti-KLH serum (Fig. 3). Consistent with the evidence from immunoblotting, the reactivity of the anti-alpha 1 antiserum in ELISA was not changed by periodate treatment.

Serological reactivity of the egg antisera against KLH also decreased following sodium periodate treatment (Fig. 4). Interestingly, the reactivity of KLH antisera for KLH increased slightly after periodate treatment (Fig. 4), an observation which is again consistent with the evidence from the immunoblot that reactivity of KLH with its homologous serum survives periodate treatment.

DISCUSSION

In this study extensive immunological cross-reactivity between KLH and *S. mansoni* egg antigens has been shown. Mild sodium periodate hydrolysis of the respective antigens has further shown that the cross-reactivity is to some extent due to carbohydrate epitopes. Periodate treatment linearizes hexose rings and thus destroys any immunological reactivity that the carbohydrate possesses, but does not alter the structure of the polypeptide chain (Bobbit, 1956; Eylar & Jaenloz, 1963; Woodward *et al*. 1985; Dunne *et al*. 1988*b*). The antigenicity of treated and non-treated antigens was compared in both ELISA and Western immunoblotting, and the use of rabbit sera raised against individual *S. mansoni* egg antigens has confirmed the presence of carbohydrate epitopes on the antigens alpha-1 and k5 in particular (Dunne *et al*. 1986; Dunne, Jones & Doenhoff, 1991; Langley *et al*. 1994). The lack of effect of periodate on the reactivity of alpha-1 in ELISA suggests that this antigen may not be as heavily glycosylated as k5 or that peptide epitopes are more dominant. The increase in intensity of reactivity of alpha-1 following periodate treatment of electoblotted antigens suggested epitopes present on this antigen may be masked by one or more carbohydrate(s).

Periodate treatment resulted in a marked decrease in the reactivity of anti-KLH antibodies against *S. mansoni* SEA and likewise, the reactivity of anti-egg antigen sera against KLH was severely ablated by periodate treatment. In both immunoblotting and ELISA KLH retained some antigenic activity with homologous antiserum after periodate treatment, due either to peptide or periodate-insensitive carbohydrate epitopes. Overall, the results indicate that much of the humoral immune response to *S. mansoni* egg antigens and to KLH in the rabbit is directed against carbohydrate epitopes. In this study total IgG responses were measured. However, Langley *et al*. (1994) demonstrated in humans that IgG2 responses were restricted to sodium periodate-sensitive carbohydrate antigens and high molecular weight antigens present in SEA. IgG1 and IgG3 responses were directed against both carbohydrate and peptide epitopes and IgG4 responses were restricted to periodate-resistant epitopes in SEA.

The usefulness of KLH as a serodiagnostic antigen for schistosomiasis has been advocated in several studies (Mansour *et al*. 1989; Markl *et al*. 1991; Alves-Brito *et al*. 1992; Yuesheng *et al*. 1994). The present study has indicated that the reason for this is most likely its high degree of immunological cross-reactivity with those schistosome egg antigens that have previously demonstrated sensitivity and specificity for detecting human *S. mansoni* infections, and in particular k5 and the 2 antigens comprising the fraction CEF6 (McLaren *et al*. 1981; Mott & Dixon, 1982; Dunne, Hillyer & Vazquez, 1988*a*; Doenhoff *et al*. 1989, 1993; Eltiro *et al*. 1992). The cross-reactive antibodies appear to be reacting mainly against carbohydrate epitopes, suggesting that alpha-1, omega-1, k5 and KLH share 1 or more common carbohydrate moieties. The exact structure of these epitopes has not yet been elucidated.

While the above studies indicated that KLH may be a useful diagnostic antigen in countries where schistosomiasis is endemic (Mansour *et al*. 1989; Markl *et al*. 1991; Alves-Brito *et al*. 1992; Yuesheng *et al*. 1994), the evidence from another study suggested that KLH may not be as useful in a non-endemic clinical setting (Verweij *et al*. 1995). Thus, Verweij *et al*. (1995) reported that KLH, when used in ELISA, could not discriminate between acute (3 month) or chronic (15 month) schistosomiasis mansoni or haematobium infections in Dutch travellers returning from Mali. Furthermore, these authors also reported that there was no significant difference in the anti-KLH response before and 1 year after chemotherapy with praziquantel (Verweij *et al*. 1995). Similar observations were described by Markl *et al*. (1991) who reported that the anti-KLH response was similar in acute and chronic patients.

KLH has also been used for the diagnosis of *S. haematobium* (Markl *et al*. 1991; Xue *et al*. 1993; Verweij *et al*. 1995) and has demonstrated better sensitivity for acute rather than chronic *S. japonicum* infections by the detection of IgG or IgM (Zheng *et al*. 1992; Cen *et al*. 1996) in ELISA, or dot-ELISA (Li *et al*. 1997). KLH shares carbohydrate epitopes with the cercarial surface, excretory/secretory antigens and egg antigens of *S. japonicum* (Yi *et al*. 1991)

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and it may be speculated that KLH also shares carbohydrate epitopes with antigens present in *S. haematobium*. In this respect the diagnostic potential of KLH is distinct from that of at least 2 of the *S. mansoni* egg antigens under consideration here, since CEF6 has been shown to be schistosome species- and life-cycle stage-specific (McLaren *et al.* 1981; Dunne *et al.* 1984) and also useful in monitoring the chemotherapeutic efficacy of oxamniquine (Dunne *et al.* 1988*a*) and hycanthone (Doenhoff *et al.* 1989).

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