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Predominance of IgA deposits in glomeruli of *Schistosoma mansoni* infected mice

A. K. EL-SHERIF* & D. BEFUS *The Host Resistance Program, Department of Pathology, McMaster University, Hamilton, Ontario, Canada*

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SUMMARY

We found that IgA is predominant among the immune deposits in the renal glomeruli of mice infected with *Schistosoma mansoni*, and thus conducted an analysis of the deposition of different immunoglobulin isotypes in the glomeruli throughout the course of infection in mice. Both immunofluorescent and immunoperoxidase methodologies were employed and compared. The abundance of *S. mansoni* antigens and the isotypes of antibodies to these antigens were examined in the sera and kidney eluates of mice during the course of infection and the results were related to the findings of immunohistopathology. Our observations suggest that at least some immune complexes form *in situ* in the glomeruli of infected mice and support a possible role of liver damage in the pathogenesis of renal disease in schistosomiasis. Intestinal mucosal immune responses to schistosome antigens may be important in the evolution of renal disease. In addition, the relevance of the murine model to human schistosomal nephropathy is questioned.

Keywords *Schistosoma mansoni* glomerulonephritis IgA mice kidney

INTRODUCTION

The presence of glomerular lesions in man and experimental animals infected with *Schistosoma mansoni* and *Schistosoma japonicum* is well established (Houba, 1979; Andrade & Rocha, 1979). Although renal involvement in schistosomiasis has most frequently been reported in association with Symmer's pipe stem fibrosis and the clinical hepatosplenic form of the disease, there is a paucity of information to explain this relationship.

An association between hepatobiliary alterations and deposition of IgA in the renal glomeruli has been reported in patients with cirrhosis (Berger, Yaneva & Nabarra, 1978) and hepatitis (Levy & Kleinknecht, 1980). Evidence that IgA or IgA-containing immune complexes are selectively transported from blood to bile by hepatocytes and hence to the intestine (Peppard *et al.*, 1981; Limet *et al.*, 1983) suggests that hepatic pathology may depress such clearance mechanisms and facilitate glomerular deposition of IgA immune complexes.

Moreover, an ongoing IgA response to a replicating agent at a mucosal surface could also be important in IgA nephropathy (Emancipator, Gallo & Lamm, 1983; Sancho *et al.*, 1983). Thus, IgA antibodies to schistosome antigens in the intestines of infected hosts (Houba *et al.*, 1976) may be relevant to the development of renal lesions. Most of the studies of renal disease in schistosomiasis have focused primarily on the role of IgG and IgM in pathogenesis. However, Van Mark, Deelder & Gigase (1977) and Digeon *et al.* (1979) consistently detected IgA among the mesangial deposits in *S. mansoni* infections of mice, but limited information is available about the prevalence of such deposits in experimental or human schistosomiasis.

Given this paucity of information, together with the difficulties reported in interpreting the significance of renal deposits in normal mice (Markham, Sutherland & Mardiney, 1973), we analysed immune deposits in the glomeruli of mice infected with *S. mansoni*. The sensitive 'Avidin-biotin complex' method (ABC), as well as conventional immunofluorescence techniques, were used to evaluate immune deposits and relate them to circulating levels of antigen and antibody during the course of infection.

MATERIALS AND METHODS

Mice infection

Female CBA/J mice (Jackson Laboratories, Bar Harbor, ME), 6–8 weeks old, were infected intraperitoneally with 60 cercaria of the Puerto Rican strain of *S. mansoni*. Equal numbers of normal, age-matched mice of the same strain were used as controls. Four to six infected and equal numbers of control mice were killed weekly from week 6 of infection.

* Present address: Suez Canal University, School of Medicine, Department of Internal Medicine, Ismailia, Egypt.

Correspondence and present address: Dr D. Befus, Gastrointestinal Research Group, Department of Microbiology & Infectious Diseases, The University of Calgary, Health Sciences Centre, Calgary, Alberta, T2N 4N1, Canada.

Starting from week 4 of infection, urine was examined weekly for protein and blood using hemacombistix strips (Miles Laboratories, Naperville, IL). For serological investigations serum samples were obtained weekly from killed groups, pooled, aliquoted and kept at −70°C.

A piece of jejunum 10 cm from the pylorus, large intestine just distal to the caecum and pieces of kidney, liver, spleen, lung and trachea, were fixed in Mota's basic lead acetate. For morphological studies, paraffin embedded tissue was processed and stained with haematoxylin and eosin, or periodic acid Schiff.

Antigens and antisera

Soluble worm homogenate (SWA) was prepared from adult *S. mansoni* from which the outer and inner bilayers of the apical membrane had been previously stripped by two incubations in digitonin solution. Worms were washed, homogenized in phosphate buffer saline (PBS) and stored at 4°C for 24 h with vigorous shaking before centrifugation at 40,000 ***g*** for 1 h. The supernatant was dialysed for 24 h against PBS, freeze-dried and reconstituted to 3·6 mg protein/ml.

Rabbit anti-SWA was prepared by five weekly subcutaneous immunizations of two rabbits with 0·25 mg protein of worm homogenate. The first dose was mixed with an equal volume of complete, and subsequent doses with incomplete, Freund's adjuvant. Serum was collected 2 weeks after the last injection, precipitated three times in a 50% solution of saturated $(NH4)_2SO_4$, dialysed exhaustively against PBS at 4°C, freeze-dried and reconstituted to 7 mg/ml. Immunoelectrophoresis showed that rabbit anti-SWA gave five precipitation lines against SWA and stained gut and parenchyma of the adult worms using indirect fluorescence.

Rabbit antisera to mouse IgG, IgM and IgA were obtained from Litton Bionetics Inc. (Kingston, MD). Rabbit antiserum to mouse IgE was obtained from Nordic Laboratories (Tilburg, Netherlands). Fluoresceinated $F(ab)_2$ fragment of goat anti-mouse C3, rhodamine labelled $F(ab)_2$ fragment goat anti-rabbit IgG (Fc fragment), HRP conjugated goat anti-rabbit IgG, fluoresceinated IgG fraction of goat anti-guinea pig C3, and the guinea pig complement were obtained from Cappel Laboratories Inc. (Westchester, PA).

Monospecificity of antisera was tested by immuodiffusion. Blocking controls used sections of mouse intestine and spleen layered with the unconjugated reagents followed by conjugated antisera.

Avidin-biotin complex (ABC technique)

Five micrometer sections of Mota-fixed, paraffin-embedded tissues were used. Endogenous peroxidase was blocked with 5% hydrogen peroxide in methanol for 30 min. Slides were rehydrated with graded ethanol and washed in PBS for 20 min before and between the different incubations. Sections were preincubated for 20 min with 1% normal goat serum in PBS and then incubated with primary antibody for 45 min. All sections were then incubated for 45 min with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) followed by a 45 min incubation with avidin and biotinylated horseradish peroxidase (HRP; Vector Laboratories). The label was developed for 5 min with 3,3-diaminobenzidine tetrahydrochloride (ICN Pharmaceuticals, Plainview, NY, 0·5 mg/ml) in 0·1 M Tris buffer pH 7·6 with 0·01% H_2O_2. Slides were counterstained with Mayer's haemalum (BDH, Toronto, Canada). Controls consisted of those without first antibody, second antibody or HRP conjugate. PBS washes occurred between different phases of the procedure.

Immunofluorescence (IF)

Immunofluorescence was performed on snap-frozen tissues sectioned using a cryostat. Slides were fixed in cold acetone (5 min) and washed three times in PBS before and after incubations (45 min) with antisera. The detection of SWA in tissues was tried before and after partial elution of kidney sections by citrate buffer at pH 3·2 for 2 h at room temperature to remove tissue bound immunoglobulins (Danno, Fukuyama & Epstein, 1979). Anti-SWA antibody was detected in tissues using SWA followed by rabbit anti-SWA antiserum and finally rhodaminated goat anti-rabbit IgG. To study whether glomerular immunoprecipitates were able to fix complement *in vitro*, cryostat sections of kidney were incubated with guinea pig complement for 1 h followed by FITC conjugated goat anti-guinea pig C3.

In both IF and ABC studies, the degree of staining was scored on coded sections as follows: 0 = none detected; ± = minimal glomerular staining; + = segmented small deposits in less than 50% of the glomeruli; 2+ = segmented small deposits in more than 50% of the glomeruli; 3+ = universal granular deposits, mostly fine; 4+ = universal granular deposits, mostly coarse (lumpy). To depict diagrammatically the changes in the amount of glomerular deposits with time, the sum of the '+' weekly scores for each group of mice was divided by the number of examined sections and the result was rounded to the nearest ±0·5 (the 'Immunofluorescence or Staining Score').

Elution of kidney tissue

A modified technique of Jeannet & Lambert (1975) was used. Pooled kidneys of the mice killed weekly were weighed, minced and washed in PBS overnight at 4°C. Fragments were homogenized and washed repeatedly at 10,000 ***g*** for 20 min in the cold until the supernatant was clear. The sediment was further extracted in 0·05 M sodium acetate pH 2·5 at 37°C for 1 h. The material was centrifuged at 10,000 ***g*** (20 min), the supernatant adjusted to pH 7 with NaOH, dialysed overnight with PBS, pH 7·2, and lyophilized. When reconstituted the supernates were brought to volumes proportionate to the original weights of the pooled kidneys (1:2 ml/g).

Serological investigations

The detection and measurement of SWA and anti-SWA antibodies were investigated in various ways: (1) Countercurrent immunoelectrophoresis as described by Houba *et al.* (1976); (2) Passive haemagglutination with formalized tanned sheep erythrocytes sensitized with either SWA or anti-SWA antibodies (200 μg protein/ml added to equal volume of cells) following Hoshino, Camrago & Da Silva (1970); (3) Enzyme linked immunoabsorbent assay (ELISA) for determination of anti-SWA antibody classes in sera, kidney eluates and cryoprecipitates of the eluates was performed in microtitre plates (Qian & Deelder, 1983; Zodda, Abdel-Hafiz & Phillips, 1983). Initially, unknowns were tested in duplicate with controls. When appropriate dilutions were identified, the samples were tested in one plate for each of the isotypes of anti-SWA antibodies so that the optical density (OD) readings could be

directly compared. Poly-L-lysine pretreated plates (NUNC, Denmark) were incubated for 24 h at 4°C with SWA (100 μg/ml). The plates were washed with 1% BSA in a 0·2 M PBS containing 0·05% Tween-20 (Bio-Rad) and coated for 1 h at room temperature with 4% BSA in 0·1 M carbonate buffer (pH 9·6). The plates were washed and test samples were added to duplicate wells. The plates were incubated for 2 h at room temperature and washed. Rabbit anti-mouse IgG, IgM or IgA were added to appropriate wells, incubated for 2 h, washed and HRP conjugated goat anti-rabbit IgG added to each well. After incubation for 2 h, the plates were washed and O-phenyldiamine (1 mg/ml) containing hydrogen peroxide (0·04%) in 0·1 M citrate buffer (pH 4·5) was added. After 30 min the reaction was stopped with 1 M H_2SO_4 and the results determined using an ELISA reader (Dynatech, Alexandria, VA). Control wells received either no antigen, no test material, no first antibody or no HRP conjugate. Reported OD were corrected by subtracting the OD of wells without antigen.

RESULTS

Mice infections and light microscopy

Infection as confirmed by counting worms recovered by perfusion of the hepatic portal vein. Gross pathology was evident in livers and spleens and the mortality rate among infected animals exceeded 50% by the 12th week of infection. The amount of proteinuria, however, was not significantly different in infected compared to uninfected mice. Under light microscopy, the morphological differences between kidneys of infected and uninfected animals were not significant.

Immunoglobulin deposits

Because our initial studies showed a high frequency of immunoglobulin deposits in kidneys of normal mice, we used a semi-quantitative analysis (Immunofluorescence or Staining Score) to depict the differences between the groups. Granular deposits were mainly mesangial, although on a few occasions, the distribution of IgM and IgG extended to the capillary walls. The frequency of mice positive for immunoglobulin deposits during infection is given in Table 1; only granular deposits were evaluated. Linear fluorescence, both segmental (streaks) and uninterrupted along the capillary basement membrane was seen in about 20% of specimens of infected and uninfected mice. Using IF, significant granular deposits of IgA and IgM were found in infected mice as compared to the controls at week 8 of infection (Fig. 1a, b). This difference was maintained for IgA throughout the infection and became more prominent after the 12th week. In contrast, for IgM deposits, the difference between infected and control animals became less prominent by the 12th week, primarily because of increased deposits in uninfected mice.

IgG deposits (Fig. 1c), mostly segmental, were seen using IF in about 50% of infected animals and were only prominent compared to controls at weeks 13 and 14. In only one animal (week 12) were pronounced (4+) IgG deposits seen. However, many infected mice had faint interstitial deposits of IgG.

Using ABC technique, linear staining was less prominent and the extent of mesangial deposition was generally more limited than on IF. However, differences between the numbers of positive and negative cases using the two techniques (IF and ABC) were not statistically significant for any of the three classes (Table 1).

Table 1. Percentage of uninfected and *Schistosoma mansoni*-infected mice with granular immunoglobulin deposits as detected by different immunohistochemical techniques

		Immunohistochemical technique	
Isotype	Mice	Fluorescence (IF) (*n*)	Peroxidase (ABC) (*n*)
IgA	Infected	72 (31)	68 (26)
	Uninfected	42 (11)	37 (7)
	*P**	<0·02	<0·05
IgM	Infected	76 (34)	82 (36)
	Uninfected	48 (13)	53 (16)
	*P**	<0·02	<0·01
IgG	Infected	53 (23)	60 (24)
	Uninfected	44 (8)	48 (11)
	*P**	>0·5	>0·5

* Chi-squared (2 × 2 contingency table) analysis of proportion of positive animals in infected or uninfected groups.

The agreement between our results with IF and ABC techniques was assessed using the paired scores given to each of 56 infected animals which were studied by both methods. When the agreement expected on basis of chance alone was ruled out (approximately 10% for each immunoglobulin class), the degree of agreement was 72·8%, 68·5% and 63% respectively for IgG, IgA and IgM. Although these degrees show that the precise numerical scores for ABC and IF were not identical, the differences between the methods were not significantly different.

Other immune reactants

While indirect IF detected segmental and focal IgE deposits in four of 25 infected and one of 25 uninfected CBA mice (mostly after the 7th week of infection), the ABC technique detected the same deposits in only one infected and none of the control CBA mice.

Using a fluoresceinated goat anti-mouse C3, mesangial deposits of C3 were found, in three infected mice (5·3%) but fixation of guinea pig complement did not occur on renal sections from any of the infected or normal CBA mice (tested by indirect IF). C3 fixation was assessed using ABC methods.

Both IF and ABC techniques failed to detect SWA or anti-SWA antibodies in any of the kidneys from CBA mice. However, partial elution of kidney sections with citrate buffer, and prolongation of incubation time with the anti-SWA antibody revealed a reproducible, finely dispersed dotty staining for antigen in the glomeruli of some sections.

Balb/c mice

In one experiment, four infected and four normal BALB/c mice, matched with CBA mice studied concurrently, were sacrificed at the 7th week of infection. In contrast to the results for CBA

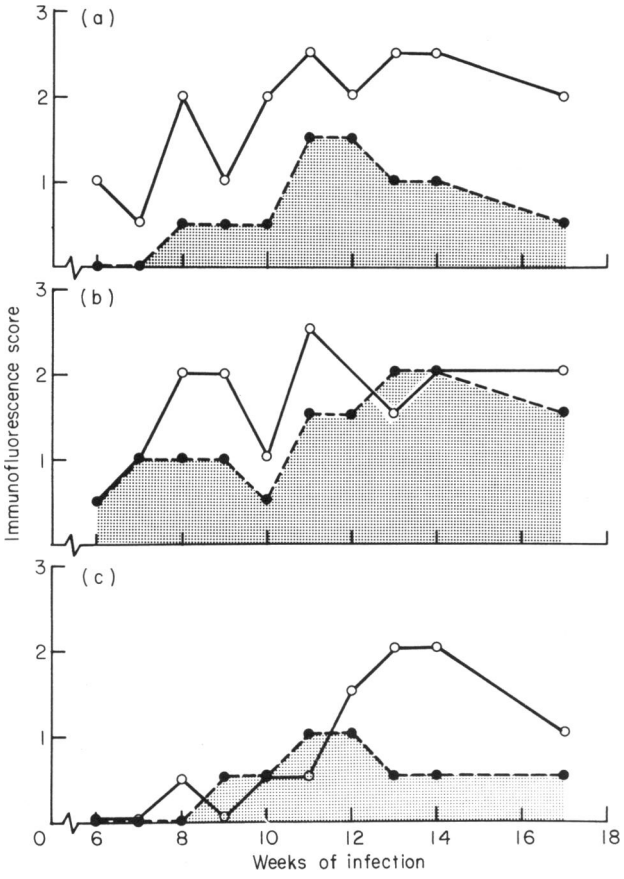

Fig. 1. Granular immunoglobulin deposits in glomeruli of CBA mice infected with *Schistosoma mansoni* (○) and in uninfected CBA mice (●). Immunofluorescent score is a semi-quantitative assessment of the distribution and abundance of deposits (0 to 4+, see Materials and Methods, $n=4$ to 6 mice per point). (a) IgA; (b) IgM; (c) IgG.

mice, IgE deposition and fixation of C3 and guinea pig complement were recognized in all four infected mice (data not shown). SWA was detected in significant amounts in one BALB/c, but anti-SWA antibody could not be demonstrated. Findings of other immunoglobulin deposits were in accordance with those of CBA mice.

Serological observations

SWA levels were studied by haemagglutination in pooled sera and kidney homogenates and are reported as the reciprocal of the highest positive dilution (Fig. 2). Antigen could be detected in the sera at the 6th week of infection and attained maximum values during weeks 8 and 11. SWA was detected in the kidney homogenates, but in lower concentrations than in sera.

Circulating levels of haemagglutinating anti-SWA antibodies appeared in sera of *S. mansoni* infected mice by week 6 to 7 of infection and persisted until week 17 without appreciable fluctuations (titres 80 to 640). Specific anti-schistosome IgM and IgG antibodies were demonstrated (ELISA) in the sera of infected mice by the 4th week of infection (Fig. 3b,c). IgG antibody levels increased thereafter, with two drops in antibody level, one at week 7 and another at week 12 of infection (Fig. 3c). Levels of IgM anti-SWA antibody increased steadily until the 9th week but fluctuated thereafter. In contrast, IgA antibody was detected in sera at the 7th week of infection and increased steadily until week 17 (Fig. 3a). In the pooled kidney eluates, IgG was the predominant isotype of anti-SWA antibody detected using ELISA (Fig. 3c). The maximum values were observed between the 9th and 13th weeks of infection. For IgA and IgM isotypes, levels were high at week 10 but then fluctuated (Fig. 3a,b).

Cryoprecipitates

Cryoprecipitates could be obtained from the kidney eluates allowed to stand at 4°C for 3 days. The cryoprecipitates from kidneys of the 10th week of infection contained anti-SWA antibody (29% of IgG, 56% of IgA and 57% of IgM was specific antibody).

DISCUSSION

Evidence from a variety of sources indicates that renal pathology in schistosomiasis is mediated by immune complexes. Whether these immune complexes are preformed in the circulation and then deposited in the mesangium, or form locally remains uncertain. The absence of urinary abnormalities or significant changes upon light microscopy in infected mice we examined, supports the view that an immunological process confined to the mesangium may not necessarily compromise renal function (Batsford *et al.*, 1979) and raises an important question about the relevance of this murine model to human renal schistosomiasis.

Most studies have focused on the role of IgG and IgM in pathogenesis, but we studied IgA, IgE, IgG and IgM equally and identified that IgA and IgM are the predominant glomerular deposits in mice infected with *S. mansoni* compared to uninfected mice. This was demonstrated using affinity purified reagents applied in both IF and ABC techniques. The agreement between the results with IF and ABC technique was high despite the conventional limitations imposed by the methodologies and the variability in glomerular involvement within kidney sections, a feature characteristic of nephropathy. The composition of the mesangial deposits in *S. mansoni* infected mice that we have identified is consistent with the findings of others (Van Mark *et al*, 1977; Digeon *et al.*, 1979) and lends support to the hypothesis that renal lesions may be related to impaired hepatic clearance of circulating immune complexes (see below).

Renal deposits of IgE have been reported in humans infected with *S. mansoni* (Shimizu *et al.*, 1976) and in monkeys infected with *S. japonicum* (Tada *et al.*, 1975), but have not been reported before in murine schistosomiasis. These deposits are of particular interest because IgE is thought to contribute to renal immunopathology by acting as a trigger for the deposition of other immune complexes (Santoro *et al.*, 1977).

The development of anti-SWA antibody and the presence of SWA in sera of infected mice were in general agreement with those results acquired by others. Worm antigens (SWA) were identified in the sera by week 6 of infection, whereas antigen was identified in kidney homogenates by week 7. The largest amount of antigen was seen in kidney homogenates at 13 weeks of infection and this was correlated with a low level of circulating antigen. Specific IgG antibodies to SWA were abundant in the sera and kidney eluates of infected mice, although IgG was not prominent in kidney sections assessed by immunohistochemistry. In contrast, IgM was a prominent glomerular deposit

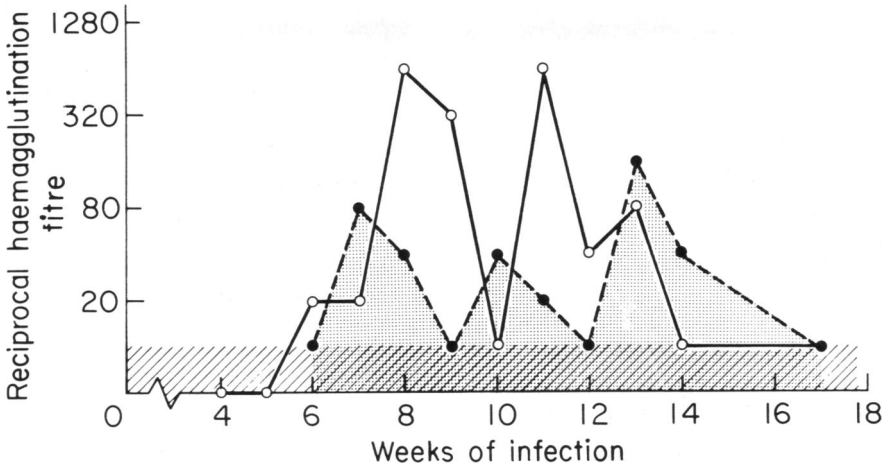

Fig. 2. Worm antigen (SWA) in sera (○) and kidney homogenates (●) of *Schistosoma mansoni* infected CBA mice; SWA detected by passive haemagglutination ($n=4$ to 6 mice/point). Diagonal shading, uninfected controls.

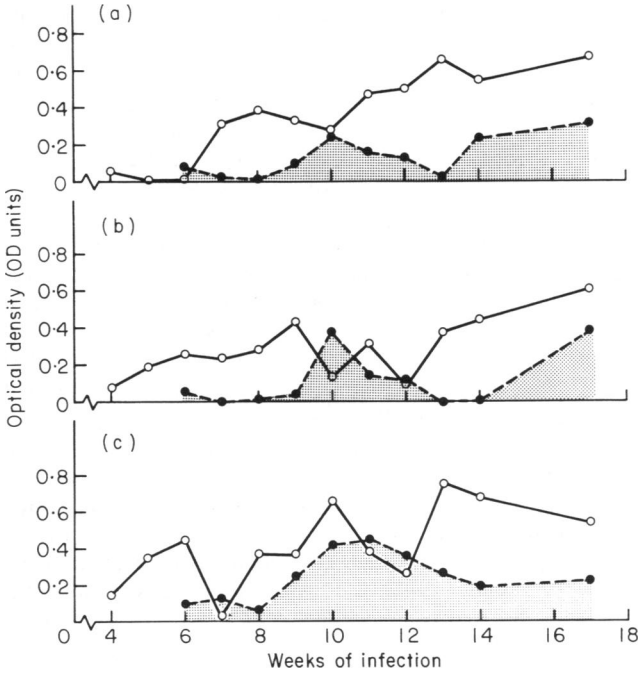

Fig. 3. Isotypes of serum and kidney eluate (●) antibodies to worm antigen in CBA mice infected with *Schistosoma mansoni*; (a) IgA, (b) IgM, (c) IgG; $n=4$ to 6 mice/point. (a) IgA; (b) IgM; (c) igG.

throughout the course of infection, but IgM antibodies were not equally prominent in the serum or kidney eluates. Despite the apparent dissociation between the isotype of circulating antibodies and those deposited in the kidney, a strong correlation has been demonstrated by others between the occurrence of glomerular deposits and the presence of circulating immune complexes in mice infected with *S. mansoni* (Digeon *et al.*, 1979). We did not look for circulating immune complexes because of their uncertain relevance in glomerular nephropathy and the limitations of current methodologies to detect IgA complexes (Border, 1983).

Earlier studies of circulating antibodies in schistosome infections (Qian & Deelder, 1983) demonstrated the prominence of specific IgG antibodies in rabbits infected with *S. japonicum*, but IgA and IgM antibodies were detected also. In contrast, Digeon *et al.* (1979) were unable to demonstrate IgA antibodies to *S. mansoni* in infected mice. Our results showed high titres of IgA antibodies specific to SWA in both kidney eluates and pooled sera. Indeed, the serum levels of IgA antibody correlated well with IgA deposition in kidney tissue. The discrepancies between the serological events and the findings in the kidney tissue for IgG and IgM in this and other reports suggest that local mechanisms and *in situ* immune complex formation may be important in the evolution of mesangiopathy of murine schistosomiasis.

The striking appearance of C3 and the fixation of guinea pig complement by sectioned glomeruli in Balb/c mice, in contrast to the paucity of complement fixation in CBA mice, suggests that significant strain differences exist. This might explain the inconsistencies among the results of different studies of murine schistosomiasis (e.g. Danno *et al.*, 1979; Digeon *et al.*, 1979).

A relationship between severity of hepatic fibrosis and renal involvement in schistosomiasis has been observed in humans and experimental animals (Von Lichtenberg *et al.*, 1971; Cavallo *et al.*, 1974), but attempts to dissociate hepatic and renal lesions have yielded conflicting results. Studies in other hepatic diseases have shown a high frequency of IgA and IgM mesangial deposits in affected individuals (Lomax-Smith *et al.*, 1983). Although impaired liver function may facilitate renal deposition of immune complexes, the reasons for the selective deposition of one immunoglobulin class or another are poorly understood. The rat liver selectively removes IgA complexes from the blood, transports these into the bile and subsequently into the intestinal lumen (Peppard *et al.*, 1981; Limet *et al.*, 1983). This pathway however has proved to be inefficient in mice (Rifai & Mannik, 1984; A. K. El-Sherif, D. Befus & B. Underdown, unpublished) where Kupffer cells of the liver and not hepatocytes are important for the uptake of IgA, IgG and IgM immune complexes (Rifai & Mannik, 1984). The human liver handles IgA and IgA-containing immune complexes in a different manner than the rat liver and thus the role of compromised hepatic function in renal deposition of IgA and IgA-containing immune complexes remains to be established.

Continued mucosal immunization with the production of

IgA antibodies can lead to renal deposition of IgA (Emancipator *et al.*, 1983). Chronic infection with *S. mansoni* and the deposition of eggs in the intestine may be a continued source of mucosal immunization and IgA antibody production. The mucosal immune response to schistosome infections has received little study but may be relevant in the formation and clearance of immune complexes and the development of renal deposits. Lastly, the properties of the relevant worm antigens may influence the clearance and organ localization of the antigen itself or circulating immune complexes. Perhaps this is one of the important conclusions that can be drawn from the results of Nash in recent studies on the fate and clearance of some of the antigens of *S. mansoni* in the mouse (Nash 1983; 1984).

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