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The role of circulating immune complexes in the glomerular disease of experimental hepatosplenic schistosomiasis

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SUMMARY

The serological and renal changes were studied simultaneously in 115 mice infected with *Schistosoma mansoni*. IgG and IgM, but not IgA anti-*S. mansoni* antibodies were detected in the sera, together with circulating immune complexes containing schistosomal antigen.

Glomerular mesangial deposits of IgA, IgM and C3 were observed. Despite the strong correlation observed between the occurrence of the circulating immune complexes containing schistosomal antigen and the glomerular deposits, results concerning the behaviour of IgA suggest that portal hypertension and liver damage have a role in the pathogenesis of glomerular lesions.

INTRODUCTION

The occurrence of glomerular lesions in human *Schistosomiasis mansoni* is now well established and the presence of immunoglobulins and complement has been demonstrated in the glomeruli of patients with hepatosplenic schistosomiasis (Andrade & Queiroz, 1968; Brito *et al.*, 1969, 1970; Silva *et al.*, 1970; Andrade, Andrade & Sadigursky, 1971; Queiroz *et al.*, 1973; Rocha *et al.*, 1976; Falcão & Gould, 1975; Hoshino-Shimizu *et al.*, 1975; 1976). Similar glomerular lesions were also observed in animals experimentally infected either by *Schistosoma mansoni* (SM) or *S. Japonicum* (Brito *et al.*, 1971; Lichtenberg *et al.*, 1971; Lichtenberg, Sadun & Bruce, 1972; Cavallo *et al.*, 1974; Hillyer & Lewert, 1974; Andrade & Susin, 1974; Natali & Cioli, 1976; Houba, Sturrock & Butterworth, 1977).

On the other hand, circulating antibodies against SM and SM antigen (SM Ag) have been found in serum (Nash, Prescott & Neva, 1974; Wilson, Sulzer & Walls, 1974; Madwar & Voller, 1975) and in the urine (Carlier *et al.*, 1975) of patients and infected animals (Berggren & Weller, 1967; Gold, Rosen & Weller, 1969; Bawden & Weller, 1974), together with circulating immune complexes (Smith *et al.*, 1975; Natali & Cioli, 1976; Bout *et al.*, 1977).

All these data suggest an immune complex pathogenesis (Dixon, 1968; Cochrane & Koffler, 1973) for the glomerular lesions observed in hepatosplenic schistosomiasis. We have examined mice experimentally infected by SM in order to study in detail the glomerular lesions and to discuss their relations with the immunological phenomena.

MATERIALS AND METHODS

Mice infection. Female C57 black mice, 8 weeks old, were infected percutaneously using the ring method (Smithers & Terry, 1965) under Nembutal narcosis. Fifty mice received a single dose of sixty cercariae of SM (Puerto Rico strain) (group 1) and sixty-five mice (group 2) were given repeated doses (three doses of twenty cercariae at 7 days interval). Sixty normal age-matched mice of the same origin were used as controls. The mice were observed weekly until week 23 after the last infection.

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Serological investigations. Serial serum samples were obtained weekly from eight to ten mice from each group. The sera were then pooled.

The detection and measurement of anti-SM antibodies were performed by haemagglutination using formalinized, sensitized sheep erythrocytes.

The immunoglobulin class of the antibodies was investigated using formalinized sheep erythrocytes sensitized with SM Ag incubated with the sera of the infected mice. Sera from the non-infected mice were used as controls. After washing, the erythrocytes were stained with fluoresceinated rabbit antisera against mouse IgG, IgA and IgM (Meloy Laboratories) and observed under a fluorescence microscope. All the sera were previously absorbed with formalinized sheep erythrocytes.

The circulating immune complexes were investigated using three different techniques: the detection of cryoglobulins, the precipitation by 3·5% polyethylene glycol (PEG test) according to the method described previously (Creighton, Lambert & Miescher, 1973; Digeon *et al.*, 1977), and the anti-complementary activity of the serum determined by the complement fixation test (CF test), performed according to Wasserman & Levine (1961) and Santoro *et al.* (1976).

The levels of IgG1, IgG2a, IgG2b, IgM and IgA were measured by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) in the sera, cryoprecipitates and PEG precipitates using immunoplates obtained from Meloy Laboratories.

An anti-SM antiserum was prepared in a rabbit by immunization with an adult whole worm extract according to the technique of Capron *et al.* (1968). An aliquot of this antiserum was fluoresceinated and used in immunofluorescence studies Another aliquot was radiolabelled with ^{125}I with chloramine T (Hunter & Greenwood, 1962) and used for detection of the SM Ag in the PEG precipitates with a technique derived from the radioimmunoprecipitation PEG assay (Santoro *et al.*, 1977): 50 μl of radiolabelled serum were added to the PEG precipitate dissolved in borate buffer (0·1 M, pH = 8·4). After 3 hr incubation at 37°C and 72 hr at 4°C, the cryoprecipitate was isolated by centrifugation (3000 *g* for 30 min), then washed five times with PEG 3·5%. The amount of bound radiolabelled antibody was evaluated by determination of the radioactivity in a gamma counter.

Urinalysis. The urine was examined weekly for the presence of protein using reactive Albustix strips (Miles Laboratories). Only the amounts of proteinuria $\geq ++$ (i.e. 1·0 g/l) were taken into consideration.

Morphological methods. Open kidney biopsies were obtained weekly from five mice from each group under anaesthesia with Nembutal. Some infected and normal mice were killed at weeks 14 and 23 and the kidneys were used for elution. Some mice from each group underwent iterative biopsies at intervals of 2 to 12 weeks.

For light microscopy, kidney, liver and spleen specimens were fixed in Duboscq–Brasil liquid, embedded in paraffin and then cut at 2·0 μ. The following stains were employed: Masson's trichrome with light green, PAS, haematoxylin and eosin. Immunofluorescence studies were performed on each kidney specimen. The renal tissue was frozen in liquid nitrogen, then cut at 2·0 μ using a Slee cryostat. Sections were treated with fluoresceinated goat antisera directed against mouse IgG, IgA and IgM (Meloy Laboratories). The monospecificity of these antisera was tested by immunoelectrophoresis and Ouchterlony double diffusion against normal mouse serum. A semi-quantitative evaluation of Ig deposits in the glomeruli was established as follows: $-$ = no significant fixation; $\pm$ = segmental deposits in some glomeruli; $+$ = small deposits in all the glomeruli; $++$ = large deposits in all the glomeruli. This estimation was based on the observation made by two independent observers. A rabbit serum against mouse C3 was prepared by the zymosan fixation method (Mardiney & Müller-Eberhard, 1965). Indirect fluorescence method was used for detection of glomerular C3 deposits.

The detection of SM Ag in renal structures was performed by immunofluorescence using SM Ag rabbit antiserum (see above). The detection was done both before and after the elution of kidney sections by citrate buffer at pH 3·2 for 4 hr at room temperature (Feltkamp & Boode, 1970). The elution of the whole kidneys from normal and infected mice was done according to Jeannet & Lambert (1975). The specificity of the kidney eluates was tested by haemagglutination on formalinized sheep erythrocytes sensitized by SM antigen.

RESULTS

Evolution of the parasitic disease

All the infected mice showed evidence of parasitic disease. After the seventh week, loss of weight and abdominal distension appeared and intestinal bleedings seemed to be one important cause of death. Mortality occurred after the seventh week and the rate increased rapidly with a maximum between weeks 8 and 11. More than 60% of mice were dead at the end of the week 14.

At the post-mortem examination, splenomegaly and white granules disseminated on the liver and peritoneum surface were observed. The microscopic study showed the presence of granulomas surrounding the parasite eggs in the liver, spleen and intestines, but not in the kidneys.

Serological observations

(1) *Antibodies.* In infected mice, serum levels of immunoglobulins (Ig) rapidly increased between week 6 and weeks 8–10 and then remained high. The proportion of IgA precipitated by PEG, expressed as a percentage of the serum level, was clearly increased in infected mice. Statistical analysis was done using the Student's *t*-test: no significant difference was observed between controls and infected mice for

PEG precipitable IgG1, IgG2a, IgG2b and IgM. On the contrary, in the case of PEG precipitable IgA both infected groups 1 and 2 differed significantly from controls (group 1: $P<0{\cdot}02$; group 2: $P<0{\cdot}05$). Fig. 1 summarizes the results obtained at week 10.

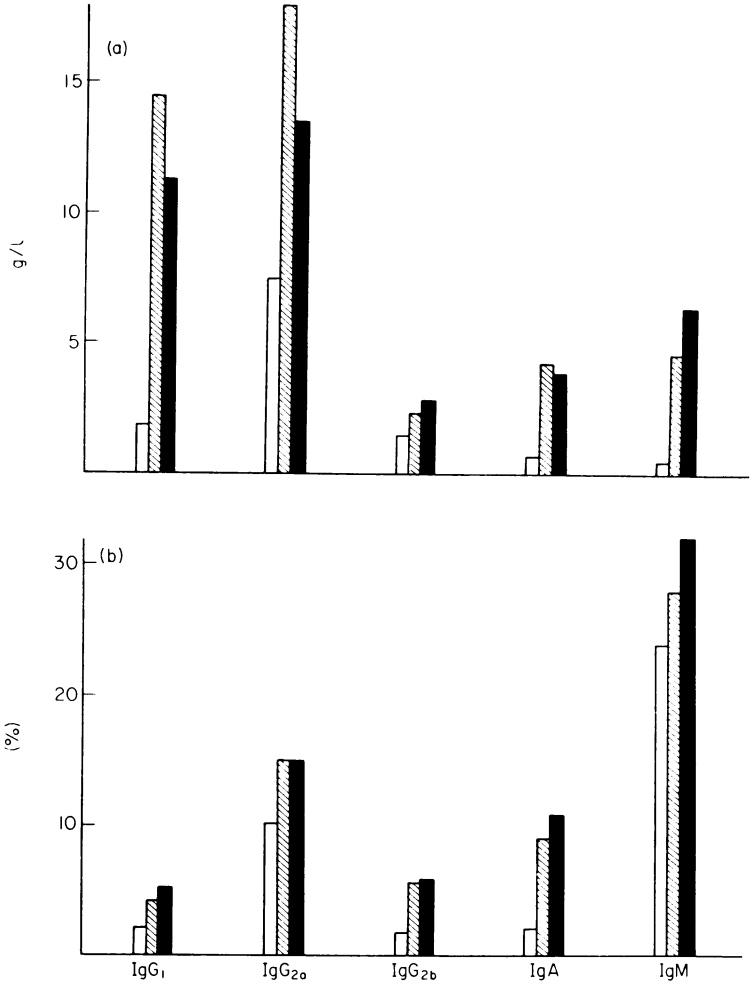

FIG. 1. (a) Immunoglobulins serum levels (week 10), (b) precipitation of the serum immunoglobulins by PEG. The amounts of precipitated Ig are expressed as a percentage of the Ig serum level (week 10). (■) Group 1, (▨) Group 2 and (□) control.

The antibodies against SM antigen appeared in the sera 6 weeks after the infection in mice of group 1 and 5 weeks after the last infection in mice of group 2. The antibody levels increased for 1 or 2 weeks and then remained high.

Anti-SM antibodies of IgM and IgG classes were found, but not of IgA. The sera from control mice always gave negative results against SM Ag.

(2) *Immune complexes.* (Fig. 2). Cryoprecipitates were found together with antibodies in the sera from infected mice. They were still present at week 23. Cryoprecipitates contained IgG, IgA and IgM. Eight weeks after infection, IgA was the principal component of cryoglobulins, especially in mice in group 2. In both groups 1 and 2, IgM appeared to be the most important component in cryoglobulins obtained at week 12. The PEG test was positive in infected mice after 6–7 weeks. The positivity increased rapidly and persisted until week 23, only decreasing in group 1. The SM Ag was searched for in the PEG precipitates from week 8 to week 13. It was detected after week 8 with highest level between weeks 9 and 10.

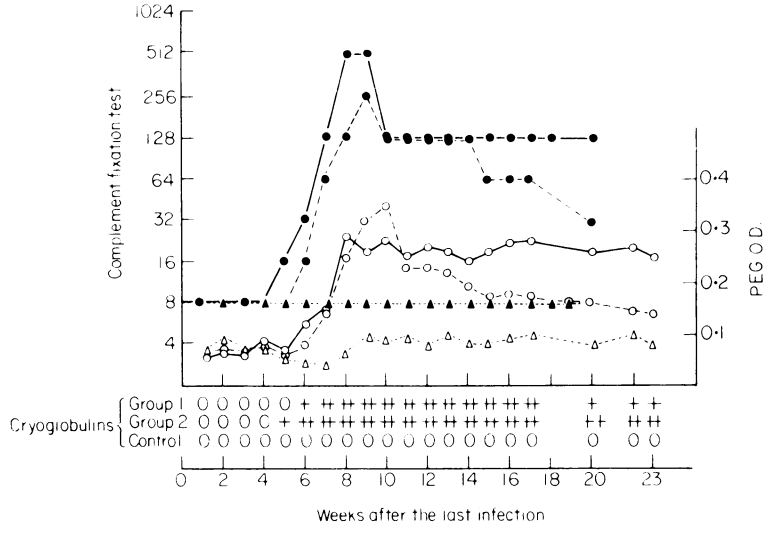

FIG. 2. Detection of circulating immune complexes. CF: Complement fixation test. The results are expressed by the reciprocal ratio of higher positive dilution. PEG: PEG test. The results are expressed by optical density. (●– – –●) CF group 1, (●———●) CF group 2, (▲. . . . .▲) CF control. (○– – –) PEG group 1, (○———○) PEG group 2, (△– – –△) PEG control.

The anti-complementary activity was present after weeks 5–6 and then increased rapidly and persisted.

Renal involvement

(1) *Proteinuria*. As soon as the fifth week, the amount of proteinuria was significantly higher in infected mice as compared to the controls. However, after 14 weeks control mice also showed proteinuria (due to ageing) and the difference between the infected and control groups did not appear to be significant.

(2) *Kidney biopsies. Light microscopy*. By light microscopy, the glomerular lesions were moderate. An increase of the mesangial matrix without mesangial cell proliferation was observed, especially in infected mice. Abnormal deposits were found in some mesangial areas. The tubules and the interstitial tissue remained normal.

Immunofluorescence results (Table 1). The results summarized in Table 1 represent the mean estimation of the semi-quantitative analysis for five mice per group.

TABLE 1. Evolution of glomerular deposits (immunofluorescence study)

Group	Type of antiserum	Weeks 6	7	8	9	10	11	12	13	14	23
Control	IgG	–	–	±	–	–	–	–	–	±	+
	IgA	–	–	–	–	–	±	–	±	±	+
	IgM	±	±	±	±	±	±	±	±	+	+
1 (60×1)	IgG	±	–	±	±	–	–	±	–	±	+
	IgA	–	+	+	±	+	+	+ +	+	+	+
	IgM	±	+	+ +	+	+ +	+	+ +	+	+	+ +
2 (20×3)	IgG	±	–	±	–	±	–	±	±	+	+
	IgA	±	+	+ +	+	+ +	+	+	+ +	+	+ +
	IgM	±	±	+	+	+ +	+	+	+ +	+	+

At the sixth week after infection, no difference was observed between normal and infected mice. In contrast, after the seventh week significant deposits of IgA and IgM were found in infected mice as compared to the controls.

Segmental and focal deposits of IgG were also present, but in the glomeruli of both infected and normal mice, whatever the time of biopsy.

On specimens obtained at the eighth week, glomerular deposits of IgA and IgM were diffuse in all glomeruli. Such deposits occupied the mesangial areas, rarely spreading along the capillary walls (Fig. 3).

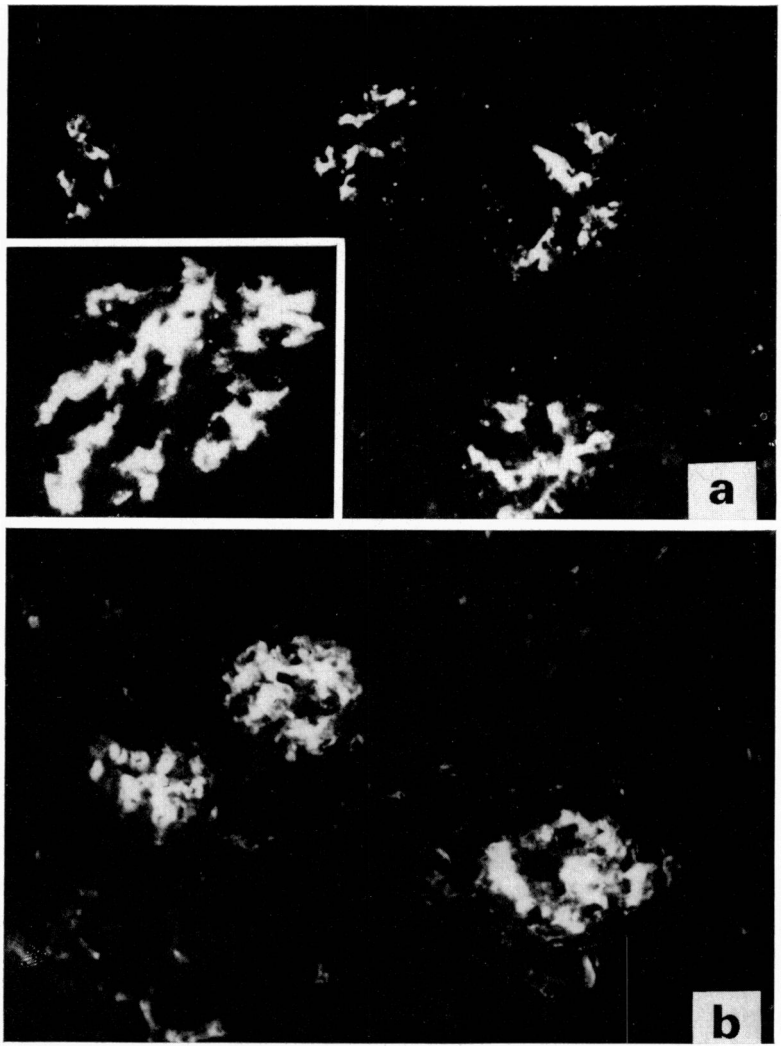

FIG. 3. Immunofluorescence. (a) Diffuse glomerular deposits of IgA. (Magnification × 225.) In the lower left quadrant: mesangial deposits of IgA. (Magnification × 360.) (Mouse from group 2 at week 8.) (b) Glomerular deposits of IgM. (Magnification × 225.) (Mouse from group 1 at week 8.)

Deposits of IgA were prominent in mice from group 2, whereas IgM was the principal component in mice from group 1. At the tenth week, the amount of IgA and IgM appeared similar in both groups 1 and 2. The glomerular deposits were still present at week 14 as well as at week 23 in infected mice. In normal mice some mesangial deposits of IgA and IgM were also found at week 23, but clearly to a lesser extent than in infected mice.

A fixation of anti-C3 serum was found in the glomeruli, together with immunoglobulins in 30% of biopsies obtained at weeks 8 and 9 and in 60% of specimens obtained at week 23, whereas no fixation was observed in biopsies obtained from controls.

Iterative biopsies were obtained in twenty-one mice at an interval of 2–12 weeks. Increased amounts of deposits were noted when an interval of more than 5 weeks was left between two biopsies.

The direct and indirect immunofluorescence method, (without previous elution of kidney sections) failed to detect SM Ag in the glomeruli (thirty-seven biopsies obtained from weeks 6–13). Conversely, after a partial elution of the sections a faint fixation of the serum against SM Ag was observed in the glomeruli in three out of nine biopsies obtained at the eighth week.

The antibody activity of eluates obtained from whole kidneys of seven normal and eight infected mice killed at week 14 was tested by haemagglutination. The test using the eluates from infected mice gave slightly, but significant, positive results, as compared to the eluates from controls.

Correlations

The correlation between the incidence of circulating immune complexes detected by the PEG test and the glomerular deposits is presented in Fig. 4. The amount of glomerular deposits increased with the positivity of circulating immune complexes. Like circulating immune complexes, the glomerular deposits were still present when mice were killed (week 23).

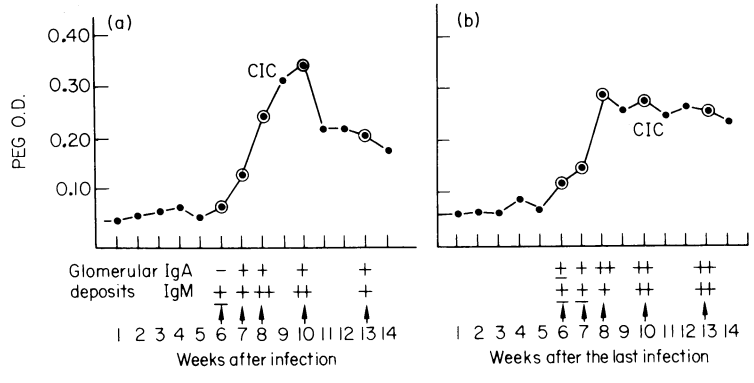

FIG. 4. Correlation between glomerular deposits and circulating immune complexes (PEG test). (a) Group 1, mice infected once; (b) group 2, mice infected three times.

DISCUSSION

It is now well established that mice experimentally infected with SM develop both serological and renal abnormalities (Natali & Cioli, 1976). As observed in man and animals (Brito *et al.*, 1970; Lichtenberg *et al.*, 1971; Cavallo *et al.*, 1974), we found in infected mice a great hypergammaglobulinaemia and an increase of anti-SM antibody levels roughly parallel to the levels of gammaglobulins. At the same time, circulating immune complexes were detectable. The three different methods used, i.e. detection of cryoglobulins, the complement fixation (CF) test and PEG test gave similar results. However, the detection of cryoglobulins and the positivity of the CF test preceded by a week the positivity of the PEG test. As free SM Ag was not precipitated by PEG at a concentration lower than 6% (Bout *et al.*, 1977), the finding of SM Ag in the PEG precipitates indicated that the immune complexes contained SM Ag. Serum IgG and IgM showed specific antibody activity against SM Ag. It is noteworthy that no specific antibody activity of IgA could be demonstrated against SM Ag, but the precipitation of serum IgA by PEG and its presence in cryoprecipitates indicated the presence of either aggregated or immune-complexed IgA.

Kidney biopsies showed the appearance of significant glomerular deposits in infected mice. These deposits contained essentially IgA and IgM and were found in mesangial areas. C3 fixation was not

constant. A similar composition of glomerular deposits was reported in SM-infected mice by Van Marck, Deelder & Gigase, 1977. Other authors have described predominant deposits of IgG, IgM and complement (Andrade & Susin, 1974; Mahmoud & Woodruff, 1975; Natali & Cioli, 1976).

In contrast with the presence of deposits detected by immunofluorescence, the glomerular changes observed by light microscopy were moderate and limited to an increase of the mesangial matrix without cell proliferation.

A strong correlation was found between the occurrence of the glomerular deposits and the presence of circulating immune complexes. Indeed, the detection of circulating immune complexes preceded by a week the appearance of glomerular deposits and both parameters had the same progression with time. Moreover, in mice from group 2, IgA was the principal component in the glomerular deposits and in cryoprecipitates at week 8, but after week 10, the ratio IgM/IgA increased in cryoprecipitates, while the deposits of IgM appeared as important as those of IgA.

Such a strong correlation suggests that glomerular deposits result from the deposition of circulating immune complexes. Several arguments from either experimental or human observations support such an hypothesis and suggest the role of SM Ag-Ab complexes. We have found SM Ag in PEG precipitates of infected mice and Bout *et al.* (1977) have made similar observations in patients with SM infection. In experimental studies, SM Ag was detected in the glomeruli using anti-whole worm extract antiserum. However, positive results were only obtained in a small proportion of animals (Natali & Cioli, 1976; Houba *et al.*, 1977). The presence of SM Ag was also demonstrated in the glomeruli in two out of twelve patients by Hoshino-Shimizu *et al.* (1976), and one case of recurrence after transplantation with the presence of SM Ag in the transplanted kidney was reported (Falcão & Gould, 1975). On the other hand, using immunofluorescence techniques, anti-SM antibody activity was found in eluates from kidneys (Hoshino-Shimizu *et al.*, 1976). We have observed only a faint fixation of anti-SM Ag antiserum in three out of nine biopsies obtained at week 8, after partial elution of the sections. Such a faint fixation should be interpreted cautiously. It is important to note, however, that kidney eluates have anti-SM antibody activity (haemagglutination test).

In any case, although all these results support the role of SM Ag anti-SM antibodies immune complexes, some observations remain unclear and are compatible with other hypotheses.

(1) Although IgG antibodies against SM Ag were present in the sera of infected mice, no significant glomerular deposits of IgG were observed, as compared to the controls. On the other hand, IgA was found in the glomeruli of all the infected mice, while no anti-SM antibody activity could be demonstrated for serum IgA.

(2) The studies of kidney involvement in human liver cirrhosis have revealed the presence of mesangial deposits of IgA, IgM and, to a lesser degree, of C3 (Nochy *et al.*, 1976; Berger, Yaneva & Nabarra, 1977). Moreover, serum levels of IgA are increased in these patients and cryoglobulins containing IgA may be found (Druet *et al.*, 1973). Thus, there is an obvious analogy between the glomerular and the serological events we found in SM infection and those observed in liver cirrhosis, especially with regard to the behaviour of IgA.

(3) Glomerular involvement is nearly exclusively observed in the hepatosplenic form of schistosomiasis. It is known that the portal fibrosis results from the presence of egg granulomas in the portal venules. A good correlation has been found in SM infection between the severity of the glomerular lesion and the degree and duration of the portal liver fibrosis (Cavallo *et al.*, 1974). Moreover, the partial ligature of the portal vein significantly increases the amounts of glomerular deposits in SM-infected mice (Van Marck *et al.*, 1977). In mice infected with single sex parasites in which no egg is produced, the occurrence of proteinuria and of glomerular deposits was obviously reduced, despite the presence of circulating antibodies against SM Ag (Natali & Cioli, 1976). Lastly, glomerular deposits of IgA and IgM are also obtained in non-infected mice with only partial ligation of the portal vein (Van Marck *et al.*, 1977). Thus, despite the evidence of circulating immune complexes containing SM Ag, the presence of liver lesions with portal hypertension is a prerequisite for the development of the glomerular lesions in SM infection. In this respect, hepatosplenic shistosomiasis may represent a model of glomerular lesions related to liver damage.

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