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SCHISTOSOMA MANSONI: ULTRASTRUCTURAL LOCALIZATION OF THE CIRCULATING ANODIC ANTIGEN AND THE CIRCULATING CATHODIC ANTIGEN IN THE MOUSE KIDNEY GLOMERULUS

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Abstract. In this study two major antigens of *Schistosoma mansoni*, the circulating anodic antigen (CAA) and the circulating cathodic antigen (CCA), were localized ultrastructurally in glomeruli of *S. mansoni* infected mice. These antigens were studied by direct gold labeling in which anti-CAA and anti-CCA monoclonal antibodies were labeled with 5 and 15 nm gold particles, respectively. CAA and CCA were demonstrable in glomeruli at week 3 in the basement membrane and from 5 weeks in moderately electron-dense material of the mesangial matrix. Both antigens were also encountered in fenestrae of the endothelial cells, in filtration slit pores, and on the luminal membranes of the epithelial cells. It appears that CAA and CCA are arrested by the glomerular basement membrane and deposited in the mesangial matrix. CAA was seen in considerably smaller amounts than CCA. This was ascribed to the fact that CAA, but not CCA, is repelled by the negative charge of the capillary walls and the glomerular basement membrane.

In *Schistosoma mansoni* infections the circulating antigens derive from the adult worm or from the schistosome egg, and are demonstrable in various fluids of the host.[1] Two of these are the negatively-charged circulating anodic antigen (CAA) and the positively-charged, neutral pH, circulating cathodic antigen (CCA).[2,3] CAA is a proteoglycan, while CCA is a heat-stable antigen with a large sugar moiety. Both are produced by the gut epithelium of adult *S. mansoni* and released into the blood of the host.[4,5]

Several studies have shown that the host kidneys play an important role in the disposal of circulating schistosome antigens from the peripheral blood.[6–13] Detailed immunohistological studies in this field have investigated the deposition of CAA and CCA in various tissues of *S. mansoni* infected mice.[8–13] By immunofluorescence both antigens were localized along the glomerular capillary walls and in the mesangium and, under the electron microscope, a number of morphologic changes were noted.[9,10] Although these results suggest that the glomerular basement membrane and the mesangium are prominent sites of uptake and processing of circulating antigens, it seems that only immunoelectron microscopic studies can shed light on the renal involvement in schistosomiasis.

For the ultrastructural localization of CAA and CCA in the gut epithelium of adult worms and other life cycle stages of *S. mansoni*, we applied monoclonal antibodies in an immunogold labeling procedure in combination with a low temperature embedding technique.[14,15] These studies allowed an accurate antigen localization combined with excellent ultrastructural detail. We used these techniques to study *S. mansoni* infection in mice at 1–10 weeks.

MATERIALS AND METHODS

Parasites and host animals

Pieces of adult male and female *S. mansoni* (Puerto Rico strain) were fixed with 2% parafor-

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maldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) at 4°C and centrifuged (5 min at 200 × *g*), resuspended in 4% gelatin at 37°C, spun down again (5 min at 200 × *g*), and cooled at 4°C. The pellets were cut into small blocks and stored overnight in 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) at 4°C. Thereafter, they were dehydrated in a graded series of ethanol at progressively lowered temperatures down to −35°C and embedded in Lowicryl K4M (Balzers, Liechtenstein) as described.[16]

Swiss outbred female mice were injected subcutaneously with 135 *S. mansoni* cercariae. Their kidneys were fixed by perfusion via the abdominal aorta with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 5 min followed by immersion of small pieces of kidney cortex in the same fixative for 3 hr at 4°C. These pieces were stored overnight at 4°C. Dehydration and embedding were performed as described above.

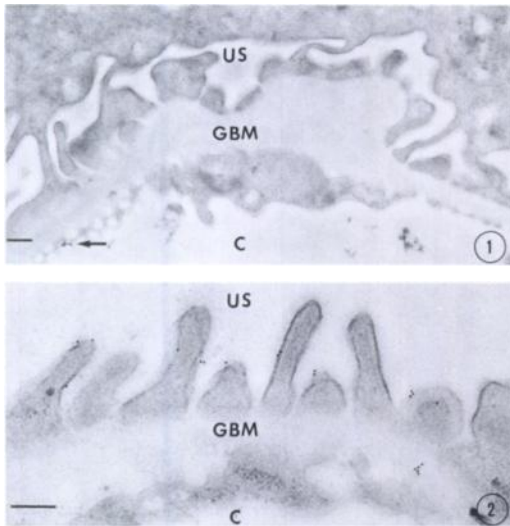
After polymerization, sections 1 μm thick were cut, briefly stained with toluidine blue, and light microscopically examined. Kidney cortex containing a glomerulus and *S. mansoni* tissue containing gut epithelium were trimmed and cut ultrathin with a glass knife. The ultrathin sections (100 nm) were mounted on 100-mesh copper grids with a hydrophilic carbon-coated collodion film, and stored until use.

Antibodies

Hybridoma cells were obtained by fusion of SP2/0 mouse myeloma cells with spleen cells of Swiss mice, which had been injected with a trichloroacetic acid-soluble fraction of adult worm antigen (AWA-TCA). As determined by an indirect immunofluorescent assay (IFA) on paraffin sections of Rossman's fixed adult male *S. mansoni*,[5] the hybridoma cell lines 54-5G10-A and 54-4C2-A secreted IgG1 antibodies which were only reactive with the schistosome gut epithelium. Their binding specificities were determined by immunoelectrophoresis against AWA-TCA and it was found that antibodies deriving from cell line 54-5G10-A were specific for CAA, and antibodies deriving from cell line 54-4C2-A were specific for CCA.[17] In order to obtain these antibodies in more substantial amounts, ascitic tumors were produced.[18] In this procedure, 1 ml hybridoma cells (1 × 10^6 cells/ml culture medium) were injected intraperitoneally in F1 BALB/c × Swiss mice. After 10 days the ascitic fluids were collected and centrifuged at 1,000 × *g* for 10 min to remove cells. Clots were removed by a subsequent centrifugation step at 10,000 × *g* for 10 min. For the immunohistochemical control experiments, serum containing polyclonal nonimmune IgG antibodies was collected from uninfected mice. The monoclonal and the polyclonal antibodies were isolated by affinity chromatography using a protein A Sepharose CL-4B column (Pharmacia Fine Chemicals, Uppsala, Sweden). Binding and elution of the IgG antibodies was performed according to the manufacturer's instructions. IgG preparations were adjusted to pH 7.0 with 0.1 N NaOH, dialyzed against phosphate buffered saline (PBS, pH 7.8), and concentrated by ultrafiltration using an Amicon PM 10 membrane (Amicon Corp., Lexington, Massachusetts) to a final concentration of 0.5 mg/ml. These preparations were stored at −20°C until use.

Preparation of IgG-gold conjugates

Uniformly-sized gold particles were prepared by reduction of tetrachloroauric acid ($HAuCl_4$; Merck, Darmstadt, West Germany) by sodium citrate or by a mixture of sodium citrate and tannic acid as described in detail elsewhere.[19] In short: gold particles 5 nm in diameter were prepared by rapidly adding a mixture of 1% sodium citrate (4 ml), 1% tannic acid (1 ml), 25 mM K_2CO_3 (1 ml), and distilled water (14 ml) to a vigorously stirred solution of 1% $HAuCl_4$ (1 ml) and distilled water (79 ml). This reaction was done at 60°C. For the preparation of 15 nm gold the same procedure was followed omitting the tannic acid. Progress of gold sol formation was visually inspected by a change in the color of the reaction mixture from brownish to red, requiring at least 5 min (5 nm gold) or 60 min (15 nm gold). Sols were heated until boiling and cooled down to room temperature on ice. For coupling of IgG to gold particles, the pH of the gold sol was always adjusted by the addition of K_2CO_3. Small amounts of a solution of 0.1 M K_2CO_3 were adequate to bring the final pH of these sols between 6 and 9. The optimal pH and the optimal amount of IgG needed to stabilize the gold sols were determined by pH- and concentration-variable isotherms according to Geoghegan and Ackerman.[20] The optimal pH was 7.5 (anti-CAA)



FIGURES 1 & 2. **1.** Electron micrograph showing a local thickening in the glomerular basement membrane in an animal at 8 weeks of infection. At the left side (arrow), 15 nm gold particles can be observed in an endothelial fenestra. GBM = glomerular basement membrane; C = capillary lumen; US = urinary space. Bar = 0.25 μm. **2.** Ultrathin section through a part of the glomerular basement membrane in an animal infected for 9 weeks. CCA is present in the glomerular basement membrane and on the luminal membranes of the epithelial cells. CAA is not detectable. GBM = glomerular basement membrane; C = capillary lumen; US = urinary space. Bar = 0.25 μm.

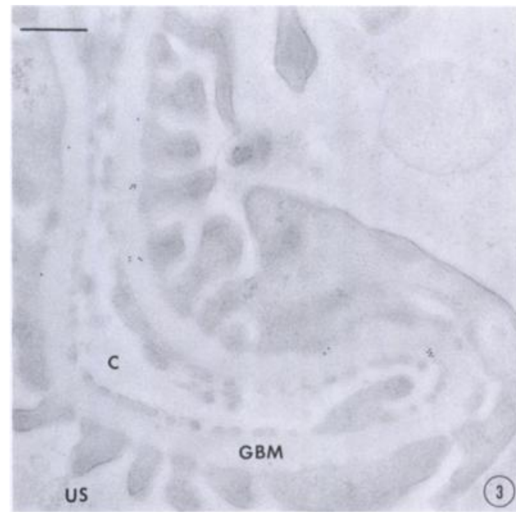


FIGURE 3. Electron micrograph showing a part of the glomerular basement membrane in an animal at 6 weeks of infection. CCA is only present in the matrix of the membrane. CAA is not detectable. GBM = glomerular basement membrane; C = capillary lumen; US = urinary space. Bar = 0.5 μm.

or 9.0 (anti-CAA and IgG from uninfected mouse serum), and for both monoclonal antibodies the optimal concentration was 20 μg/ml of gold sol. The IgG preparations were dialyzed overnight against either 1 mM phosphate buffer pH 7.5 (the anti-CCA monoclonal antibody and the polyclonal antibody preparation) and then centrifuged at 50,000 × g for 30 min, using supernatant for conjugation. As determined by the adsorption at E 280 nm, 1 mg antibody was added dropwise to 50 ml gold sol, which had been adjusted previously to pH 7.5 or pH 9.0. After 5 min, 250 μl of a 10% aqueous solution of polyethyleneglycol (PEG M_r 20,000; Serva, Heidelberg, West Germany) was added. The gold sol was then layered on a 5 ml cushion of 10% glycerol in 2 mM phosphate buffer pH 7.8, centrifuged at 125,000 × g (5 nm gold) or at 20,000 × g (15 nm gold) for 60 min. The loose part of the dark red pellet was collected, dialyzed overnight against 50% glycerol in PBS, and stored in siliconized cups at −20°C until use.

Immunohistochemistry

For the demonstration of CAA and CCA in the ultrathin sections, a double gold staining procedure was performed using an immunostain for CAA with 5 nm gold followed by an immunostain for CCA with 15 nm gold. This procedure was as follows: the grids with the sections were preincubated for 10 min at room temperature on 20 μl drops of PBS containing 1% (w/v) bovine serum albumin (PBS-BSA). Thereafter, they were incubated for 60 min at 37°C with the anti-CAA monoclonal antibody labeled with 5 nm gold (1:60 diluted). The grids were then washed three times on 20 μl drops of PBS-BSA and incubated for a second 60 min at 37°C with the anti-CCA monoclonal antibody labeled with 15 nm gold (1:30 diluted). These dilutions were optimal, giving a strong specific labeling without background staining. Controls were incubated under similar conditions, substituting the gold conjugate of the first or the second incubation with polyclonal nonimmune IgG antibodies labeled with 5 or 15 nm gold particles, respectively. For the demonstration of IgG and IgM antibodies at the ultrastructural level, commercially available gold conjugates were used (dilution 1:30): goat anti-mouse IgG antibodies and goat anti-mouse IgM antibodies coupled to gold particles with an av-

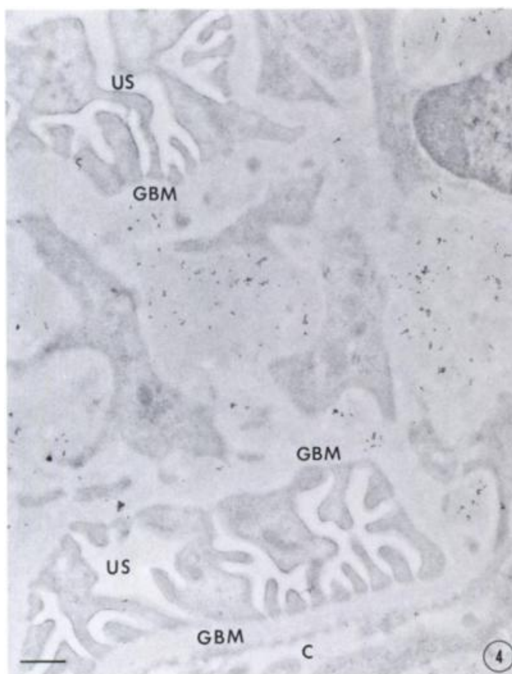


FIGURE 4. Electron micrograph showing a part of the glomerular mesangium in an animal at 9 weeks of infection. CCA is detectable in moderately electron-dense material of the mesangial matrix. CAA is not seen. At this magnification the 5 nm gold particles are too small to be distinguishable. GBM = glomerular basement membrane; C = capillary lumen; US = urinary space. Bar = 0.5 μm.

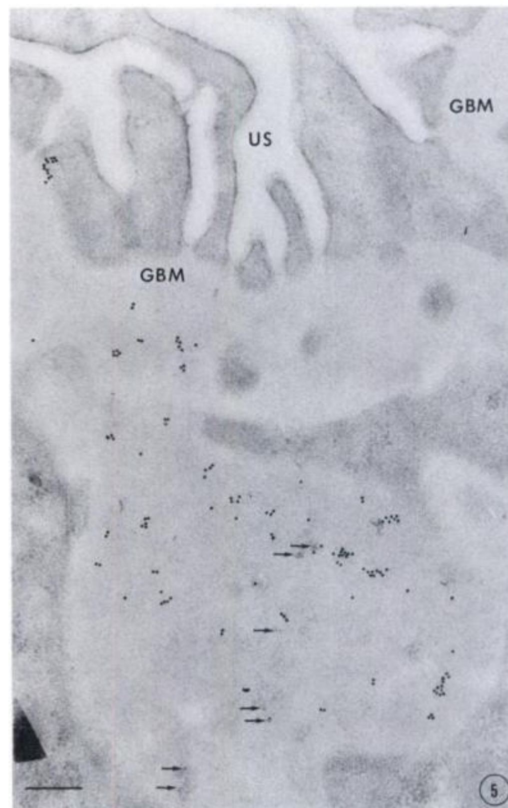


FIGURE 5. High magnification electron micrograph of a part of the glomerular mesangium in an animal at 9 weeks of infection. CCA is apparent near an epithelial slit pore and in moderately electron-dense material of the mesangial matrix. As shown by the presence of 5 nm gold particles (arrows) CAA is also seen. GBM = glomerular basement membrane; US = urinary space. Bar = 0.25 μm.

erage diameter of 10 and 5 nm, respectively (Janssen Life Sciences Products, Beerse, Belgium). Finally, the sections were rinsed with distilled water, briefly stained with uranyl acetate and Reynold's lead citrate (2 min each), and examined with a Philips EM 300 electron microscope, operated at 80 kV.

RESULTS

Glomerulopathy

A number of slight glomerular lesions similar to those described by Van Marck et al.,[9] were observed in kidneys of *S. mansoni* infected mice. During the course of the infection the number of mesangial cells slightly increased and, occasionally, local thickenings of the basement membrane were observed (Fig. 1). The mesangial matrix increased in size, developing an extensive network of homogeneous moderately electron-dense material. Glomerular lesions were obvious from the third week onward, but they were most prominent at 10 weeks after infection. No alteration of the endothelial cells was observed.

Immunohistochemistry

CCA was detectable only in the kidney glomerulus and not earlier than 3 weeks after infection. From this point onward a few 15 nm gold particles were occasionally observed in the fenestrae of the endothelial cells (Fig. 1), in the filtration slit pores, and on the luminal side of the epithelial cell membrane (Fig. 2). The amount of 15 nm gold particles found on the basement membrane varied with the duration of the infection. It was relatively low at week 3 but in-

creased up to 6 weeks after infection (Fig. 3). Thereafter, it decreased and at the end of the experiment no gold label could be detected at this location. From 5 weeks onward, CCA was associated with moderately electron-dense material of the mesangial matrix. In this compartment of the glomerulus the 15 nm gold labeling was relatively low at week 5, but increased with the duration of the infection. A strong labeling was observed at 10 weeks (Figs. 4, 5).

As shown by the presence of 5 nm gold particles, CAA was confined to the kidney glomeruli and only present after 3 weeks of infection. At intervals beyond 3 weeks CAA could be detected ultrastructurally only in trace amounts in the same areas as CCA (Fig. 5).

IgG and IgM antibodies were demonstrated at 5 weeks after infection and onward, but only in the electron-dense deposits of the mesangial matrix. The amount of gold label encountered was too low to perform double labeling experiments needed to test for the presence of circulating antigen.

Controls

No gold label was encountered in any experiment. Evidence for the specificity of the cytochemical reactions was obtained by experiments with ultrathin sections of adult *S. mansoni*. In these experiments, as with indirect gold labeling,[14,15] high amounts of CAA and CCA were seen in the same cellular compartments of the schistosome gut epithelium.

DISCUSSION

Our results show that CCA is deposited in the basement membrane, in the mesangial matrix, and on the luminal membranes of the epithelial cells. CAA was encountered at the same locations, but in considerably lower amounts. Since in the blood circulation of the host CAA and CCA are present in relatively high concentrations, these data strongly suggest that only CCA can easily pass across the glomerular epithelium and penetrate into the glomerular basement membrane. At these locations CAA was demonstrable in considerably smaller amounts, probably due to the polyanions present in the capillary wall and the glomerular basement membrane.[21,22] It is known that these polyanions induce electrostatic forces, which repulse anionic but not cationic molecules.[23–25] Biochemical experiments have shown that CAA, when injected into infected mice, is predominantly taken up by the liver and then may appear in the urine as nonantigenic material of low molecular weight.[26]

The kidney glomerulus is not only a barrier for negatively-charged substances, but also serves as a molecular sieve[27] which CAA and CCA can not pass. We observed both antigens predominantly in the mesangial matrix and only scarcely in the urinary space, filtration slit pores, and luminal side of the epithelial cell membrane. In natural and experimental *S. mansoni* infections CAA and CCA are immunogenic and give rise to specific antibodies and circulating immune complexes,[4] hence their presence in the glomerular mesangium. Immunofluorescence studies have shown CAA and CCA in the same compartment as immunoglobulins and components of the complement system.[7–9] Thus it seems likely that most of the CAA and CCA in the glomeruli is immune complex-related. Only trace amounts of immunoglobulins were demonstrated, probably due to the fixation and embedding techniques used. This point needs further study. The results of the present study only clearly show that CAA and CCA are deposited in moderately electron-dense material of the mesangial matrix. The continuous metabolic activity of the schistosome worms is reflected by the fact that at this location the amount of these antigens increased with the duration of the infection.

The changes observed in our *S. mansoni* infected mice, i.e., the generation of an extensive network of moderately electron-dense material along the glomerular capillary wall and in the mesangial matrix, are similar to those described in unisexual infected mice subjected to partial or total ligation of the portal vein.[9–11] Artificial partial ligation has been shown to be nearly as effective for the deposition of several antigens and immunoglobulins in glomeruli as the portal vein obstruction observed in bisexual infections.[9] CAA and CCA located in the kidney glomerulus most likely have escaped hepatic clearance, having been deposited after saturation of the Kupffer cells.[12,13]

CAA and CCA can ultrastructurally be demonstrated in the kidney glomerulus of the host from 3 weeks after infection. These antigens are arrested by the glomerular basement membrane and deposited in an electron-dense network of the mesangial matrix. In all probability, these antigens form part of immune complexes which

are either preformed in the blood circulation or formed in situ after binding to the capillary wall and the glomerular basement membrane. The significance of CAA and CCA in the genesis of glomerulopathy is unclear under the present experimental conditions, since it is known that other factors associated with *S. mansoni* infections, such as portosystemic collaterals, induce renal pathological changes.[9–11]

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