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Immunoelectron microscopic study of plasma protein pathways through the abnormal intestinal vasculature of $HgCl_2$ induced immune disease in brown norway rats

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Summary. Localization of immune deposits (ID) and the pathway of circulating serum proteins through the intestinal vasculature have been studied in 40 Brown Norway (BN) rats poisoned by mercuric chloride, using anti-peroxidase IgG as tracer. ID were found in all vessels but were initially detected along the epithelial basement membrane of villi and in pericytic venules and veins. ID were found in all the layers of the vessel walls. In pericytic or myocytic vessels, no ID were detected outside the adventitial lamina densa. ID trapped non immune IgG. Abnormal pathways were only found in venular capillaries and in pericytic venules with large gaps between endothelial junctions. ID were particularly abundant in these vessels.

Key words: Immunoelectron microscopy – Transvascular pathway – Experimental antoimmune disease

Introduction

Mercuric chloride ($HgCl_2$) induces immunological disease in the BN rat. The first phase is characterized in the kidney by a linear glomerular fixation of IgG and circulating anti-basement membrane antibodies. The second phase evokes an immune complex type disease (Sapin et al. 1977; Druet et al. 1978) with granular fixation of IgG in glomeruli and in the vessel walls in many organs (Bernaudin et al. 1979). The main purpose of this work was to study the pathway of plasma molecules when abnormal immune deposits are present in the vessel walls. Using anti-peroxidase IgG as tracer we studied: firstly the pathway of circulating serum protein through the different vessels compared with that observed in the normal rat (Hinglais et al. 1982), secondly the precise location in these vessels of immune deposits,

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and finally the interaction of the tracer with the deposits in small intestine of these animals. In this paper we used the nomenclature for small vessels used by Simionescu (1978a).

Materials and methods

Animals. Forty-four BN rats (Centre National de la Recherche Scientifique, Centre de sélection et d'élevage des animaux de laboratoire (CSEAL), Orléans, France) weighing 150–180 g were used in this study.

Mercurial intoxication. Rats were injected subcutaneously three times a week with 0.10 ml per 100 g of body weight of a 1 mg per ml solution of $HgCl_2$ in distilled water.

Anti-HRP antiserum. The anti-HRP antiserum was raised in a sheep immunized with HRP (Grade I Boehringer) as previously described (Druet et al. 1978). The antiserum was tested with HRP by Ouchterlony's technique and by immunoelectrophoresis using a rabbit antiserum raised against whole normal sheep serum. Precipitation lines were stained after incubation with HRP. It was thus shown that the anti-HRP antibodies were IgG antibodies. The antibody was used by intravenous injection of 1 ml/100 g rat body-weight.

Experimental procedure. Three groups of rats were given an i.v. injection of anti-HRP antiserum either 3 min (group A), 15 min (group B) or 60 min (group C) before being killed.

In group A, 20 rats were sacrificed 13 to 30 days after the beginning of $HgCl_2$ intoxication. Group B included 5 rats killed 17 days and 3 rats killed 30 days after the beginning of intoxication. In group C, 12 rats were sacrificed from 19 to 40 days after the beginning of intoxiation.

Laparotomy was performed in rats anesthetized by intraperitoneal injection of 5 mg Nembutal per 100 g body weight (Abbot Laboratories).

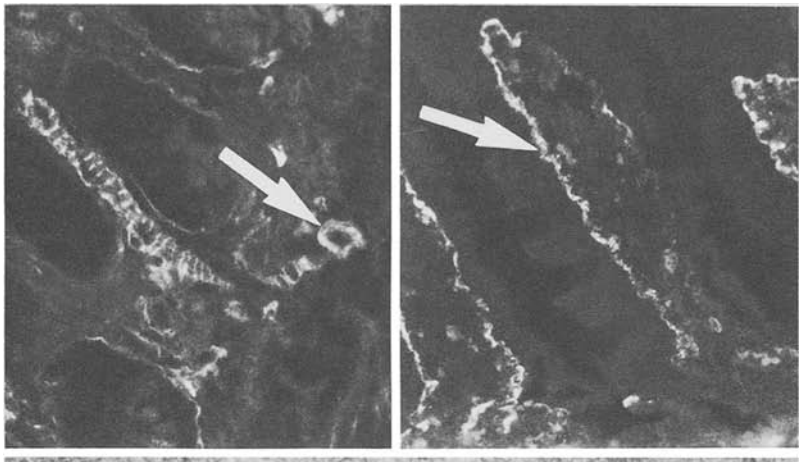
Immunofluorescence study. Segments of small intestine were snap-frozen in liquid nitrogen. Four µm thick sections were cut in a Bright's cryostat, (Huntingdon England). They were then incubated for 30 min in a moist chamber with a fluoresceinated sheep antiserum as previously described (Sapin et al. 1977).

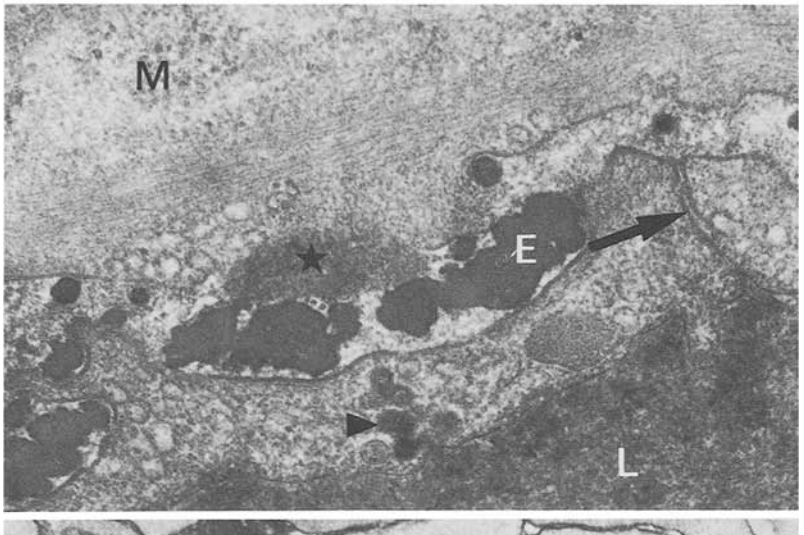
Immunoelectron microscopy. In situ fixation of a loop of small intestine was performed as described by Simionescu et al. (1972) for 10 min, then the excised intestinal loop was left in the fixative for 2 h.

The fixative used was a 2% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4. Specimens were washed for 48 h in several changes of 0.1 M phosphate buffer, pH 7.4, at 4° C. The small intestine was first cut longitudinally with a razor blade into two halves. The halves were cut into transverse sections 3 mm wide and several mm long. These strips of tissue were then cut transversly with a Smith and Farquhar tissue sectioner (Sorvall TC2) into 100 µm sections which were incubated for one hour in a 0.5 mg/ml solution of HRP (Boerhinger, grade I) in 0.1 M phosphate buffer, pH 7.4, with gentle agitation at room temperature. These sections were washed three times for 10 min each time in phosphate buffer then 10 min in tris-Hcl buffer 0.2 M pH 7.6. HRP was demonstrated using a two step procedure. The sections were first incubated in a freshly prepared solution of 20 mg of 3,3′-diaminobenzidine (DAB), (Sigma, grade II) in 10 ml of 0.2 M. Tris-HCl buffer, pH 7.6, for 45 min at room

Fig. 1. Immunofluorescence. Day 20. Fluoresceinated sheep antirat IgG antiserum. Vessels in submucosa and lamina propria of the small intestine. A pericytic venule shows regular bands of staining. A section of a small vein or artery is also labeled (*arrow*). (× 700)

Fig. 2. Immunofluorescence. Day 13. Same antiserum as Fig. 1. Intestinal villi. The base of the epithelium is labeled with a continuous granular pattern (*arrow*) (× 700)





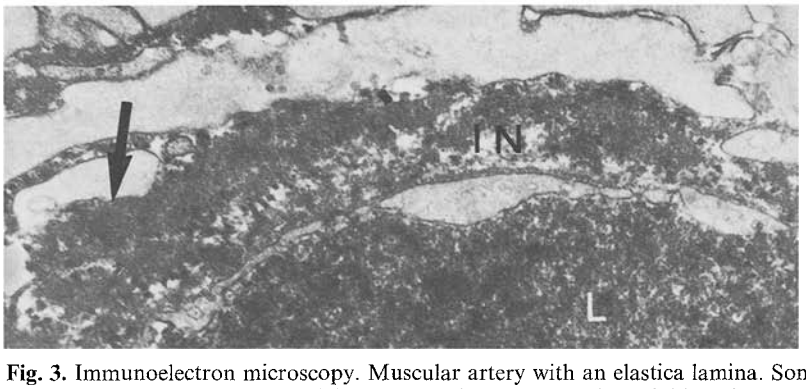


Fig. 3. Immunoelectron microscopy. Muscular artery with an elastica lamina. Some intracytoplasmic endothelial vesicles are labeled (*arrowhead*). Interendothelial junction is unlabeled (*arrow*). An abnormal cluster of heavily impregnated material is stuck on the myocitic plasma membrane (star) (× 48,000)

Fig. 4. Immunoelectron microscopy. Fenestrated capillary. The interstitial space (*IN*) is strongly impregnated by reaction product as is capillary lumen (*L*). Some masses (*arrow*) may correspond to the abnormal deposits. (× 30,000)

temperature, then incubated for 15 min in the same solution containing H_2O_2 at a concentration of 0.001%. The sections were washed in 0.2 M phosphate buffer, pH 7.4, and post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 15 min. They were dehydrated in a graded series of alcohols and embedded in Epon 812. In 6 rats, from group C (sacrificed on day 19, 20 and 22) an additional procedure was performed as described by Simionescu and Simionescu (1976) using tannic acid. After incubation in DAB-H_2O_2 medium, sections were fixed in 2% osmium tetroxide in 0.2 M cacodylate buffer pH 7.4 for 15 min. Sections were then incubated in a 0.5% tannic acid solution in 0.2 M cacodylate buffer for 1 h and washed in several baths of cacodylate buffer for 18 h. Dehydratation and embedding in Epon were performed as usual.

Ten to 15 semi-thin sections were obtained from each rat for examination by light microscopy (LM). Appropriate areas were then cut into thin sections.

Controls. 1. Small intestines of BN rats that had received a subcutaneous injection of distilled water instead of $HgCl_2$, were examined by immunofluorescence using fluoresceinated antirat IgG antiserum.

2. Intestine of normal rats were previously examined for normal permeability to plasma proteins (Hinglais et al. 1982).

3. The intestines of two BN rats injected with anti-HRP antiserum were treated as above; the specimens were not incubated with HRP but only with DAB-H_2O_2 in order to test for endogenous peroxidase activity.

4. Two other normal rats were processed by the complete technique to detect non specific trapping of peroxidase in fixed tissue.

Results

Immunofluorescence

Granular fluorescence was detectable in pericytic venules at day 17 and was plentiful after day 19 (Fig. 1). Between day 19 and 22 granular fluorescence was seen in arteries and veins in the submucosa. This granular fluorescence was more abundant after day 20. Linear and granular fluorescence was detectable along the epithelial basement membrane of the villi at day 14 and was heavy after day 16 (Fig. 2).

Immunoelectron microscopy

In group A, only the vascular lumens were labeled. In group B and C the vascular walls and extracellular spaces were also stained.

Fig. 5. Immunoelectron microscopy. Large vein. A large labeled vesicle appears to cross the thin endothelial cytoplasm (*arrow*). Tannic acid (× 70,000). *L*, Lumen; *M*, Muscular cell

Fig. 6. Immunoelectron microscopy. Large vein. There is an obvious gap between two endothelial cells (*arrow*). The subendothelial space is specially heavily blackened. (× 20,000). *M*, Muscular cell

Fig. 7. Immunoelectron microscopy. Venular capillary. There is a large gap (*arrow*) between endothelial cells filled up by heavily impregnated material. An abnormal deposit (*arrowhead*) is elongated over the endothelial basement membrane (× 40,000). *L* Lumen

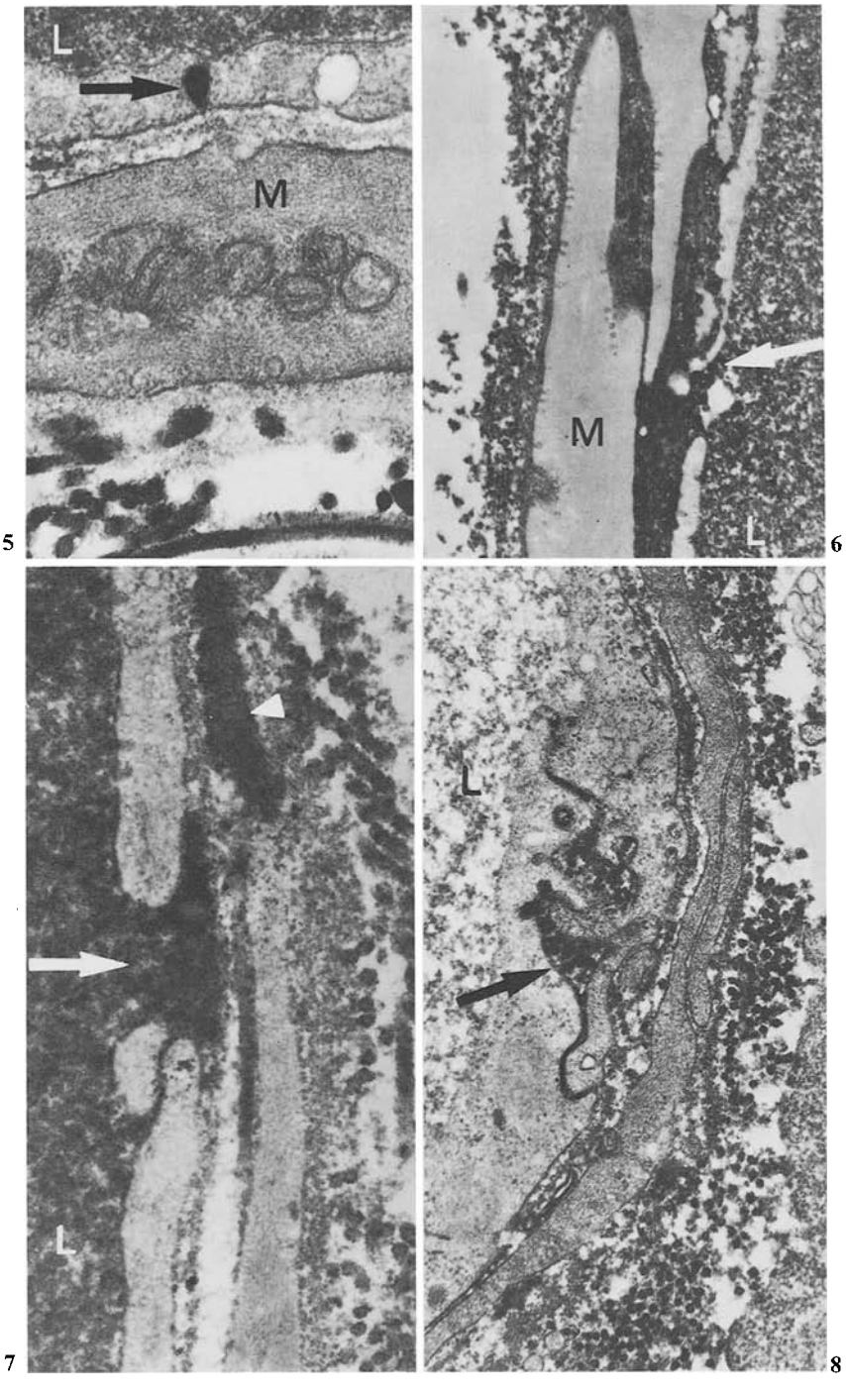


Fig. 8. Immunoelectron microscopy. Pericytic vein. There is a gap filled up by labeled material in the middle of an interendothelial junction (*arrow*) Tannic acid. (× 28,000)

a) Transvascular IgG pathways. The pattern of distribution of the reaction product was identical to that found in normal rats in arteries, fenestrated capillaries, muscular arteriolar capillaries and veins with an elastica lamina: in arteries, the intima was generally poorly labeled. Some labeled endothelial vesicles were visible in the endothelial cytoplasm (Fig. 3). In fenestrated capillaries, there were no differences in intensity of reaction product between the lumen and the adjacent interstitial space (Fig. 4). In some muscular arteriolar capillaries, a few interendothelial junctions were marked by reaction product. In veins with an elastica lamina, many endothelial vesicles were labeled. Endothelial cytoplasm was sometimes very thin with occasional vesicles which seemed to traverse the whole thickness of the cell (Fig. 5). In rare cases, gaps between endothelial cells were observed (Fig. 6). Abnormal pathways were observed only in venular capillaries and in pericytic venules. Abnormal large gaps were observed between endothelial junctions in venular capillaries (Fig. 7). In pericytic venules, interendothelial junctions were sometimes enlarged in the middle of their course (Fig. 8).

b) Localization of deposits. In vessels with myocytes (arteries and veins) or pericytic cells (pericytic venules), deposits were present throughout the whole thickness of the vessel wall. They were scattered, ovoid masses, 0.2 μm in diameter, and were more heavily impregnated by reaction product than were vascular basement membranes (Figs. 3, 9). They were observed more particularly around myocytic or pericytic cells (Figs. 9, 10) often in the loose membranoid material (Fig. 11), sometimes stuck on the plasma membrane of the cell and covered by the pericellular basal lamina (Figs. 3, 10). Deposits situated on the adventitial face of these vessels were always in this latter situation (Figs. 9, 10). In the intima, deposits were generally situated between the elastica lamina and the myocytic and/or pericytic cells (Figs. 3, 10). In fenestrated capillaries, there was a heavy impregnation of interstitial spaces by reaction product so that abnormal deposits were difficult to identify. In some cases, large irregular suspicious masses were seen, mixed with collagen fibers (Fig. 4). In muscular arteriolar type capillaries, deposits were scanty and small. They were found either inside or outside the endothelial basement membrane and also between the endothelial cell and the pericyte (Fig. 12). In the venular type capillaries, deposits were especially abundant and voluminous. They pushed away endothelial cells (Fig. 13) and encircled endothelial and pericytic cells.

Fig. 9. Immunoelectron microscopy. Arteriole. Many small round deposits, strongly labeled by reaction product, are observed mainly along the external face of the muscular cytoplasm and covered by the basement membrane (*arrows*). Some other ones are situated deeply in the vascular wall, in the intercellular spaces (*arrowhead*). (× 22,500)

Fig. 10. Immunoelectron microscopy. Pericytic vein. Three deposits (*arrowheads*) are stuck on the pericytic cytoplasm on their intimal as well as on their external face. Tannic acid. (× 38,000)

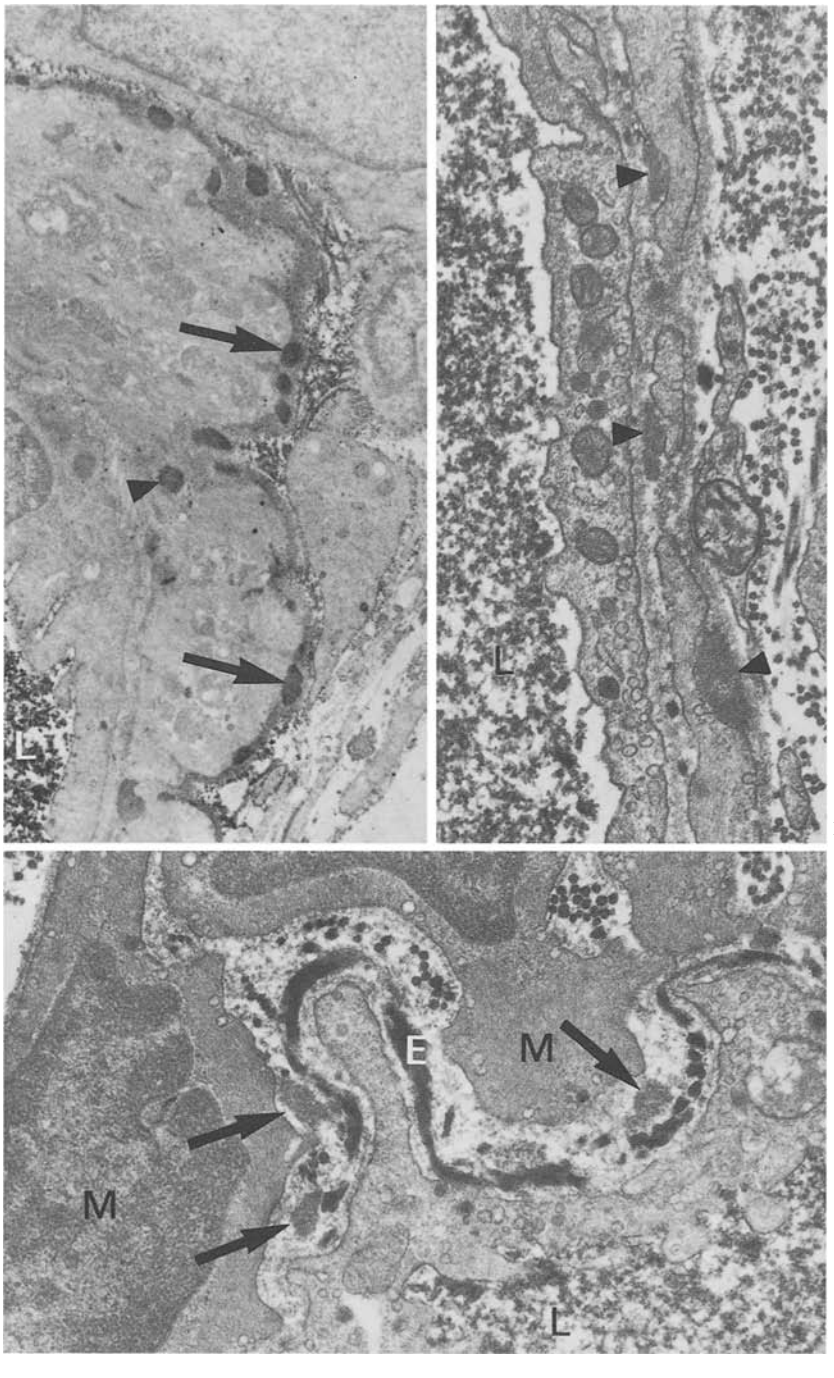


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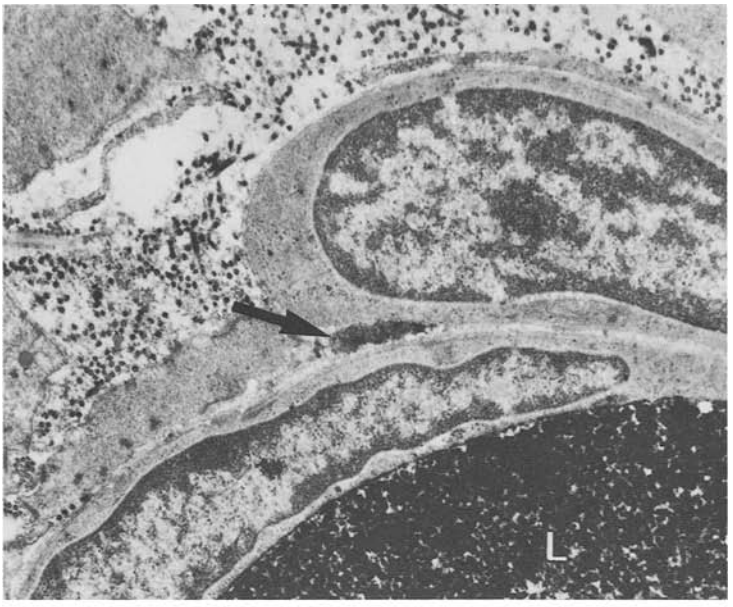
Fig. 11. Immunoelectron microscopy. Vein with an elastica lamina (*E*). Abnormal deposits (*arrow*) are situated in the subendothelial space, in the loose membranoid material between the myocite and the lamina elastica (*M*) Muscular cell. (× 21,000)



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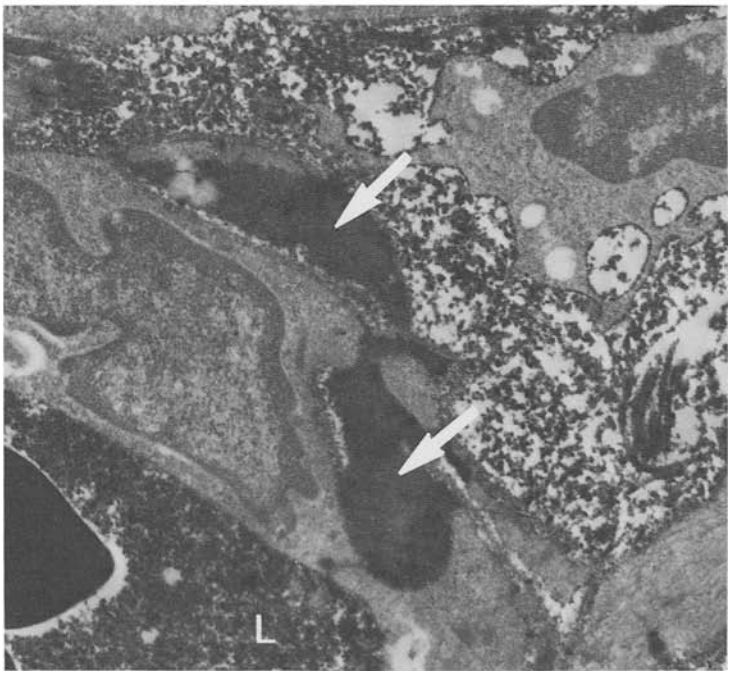

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Fig. 12. Immunoelectron microscopy. Arteriolar capillary. A small abnormal strongly impreganted deposit (*arrow*) is observed between the endothelial cytoplasm and the internal face of a pericytic cell. (× 18,000)

Fig. 13. Immunoelectron microscopy. Venular capillary. Large masses of abnormal material (*arrows*) are lying between endothelial cells and push away pericytic cells *L*, Lumen. (× 32,000)

c) Controls. 1. No immunofluorescence staining was seen in the BN rats injected with distilled water.

2. The findings in normal rats were reported in a previous paper (Hinglais et al. 1982).

3. Intestines from rats injected with anti-HRP antiserum that were treated with DAB-H_2O_2 omitting incubation with HRP were negative.

4. Non injected rats whose intestines were processed by the complete technique did not show non specific trapping of peroxidase in fixed tissue.

Discussion

This work concerns the study of intestinal vascular permeability in an immune disease induced by $HgCl_2$ poisoning in BN rats and distinguished by widespread vascular IgG deposits along the second phase of the disease (Sapin et al. 1977; Druet et al. 1978; Bernaudin et al. 1979). A protein tracer, sheep anti-HRP IgG had been chosen and the same protocol, using small intestinal vasculature, had been used as in a previous study concerning permeability of the normal rat vasculature (Hinglais et al. 1982). Results have shown that: 1. Deposits in the villi appear first along the epithelial basement membrane, later in the pericytic venules and larger vessels of the submucosa. 2. Deposits are scattered along the parietal wall of all types of vessels: in the intima as well as on the external face of pericytic or smooth muscle cells surrounded by the external basal lamina. In all cases, adventitial basal lamina of pericytic or myocytic cells appears to be a barrier to the diffusion of the deposits. 3. Qualitative modifications of pathways are clearly observed only in venular capillaries and pericytic venules with the observation of gaps between endothelial junctions. 4. Deposits trap circulating immunoglobulins. These results suggest that abnormal material reaches the vascular wall from the lumen and not from the interstial space because the deposits are especially abundant in vascular segments known for their higher permeability (fenestrated capillaries) and for the lability of their interendothelial junctions, i.e. venular capillaries and pericytic venules (Simionescu et al. 1978). The presence of deposits in the intima of muscular arteries without obvious changes in endothelial junctions supports the possibility of permeability through the endothelial cytoplasm of this type of vessel under normal conditions. In these relatively large vessels as in pericytic venules, the adventitial basement membrane acts as a barrier to the diffusion of immune material. Similar observations have been made by Simionescu et al. (1978 b), in veins after histamine infusion and a peroxidase tracer. The trapping of non immune tracers in immune IgG deposits has already been shown in experimental glomerulonephritis (Schneeberger et al. 1974, 1979). The same phenomenon occurs with more physiological tracers such as heterologous immunoglobulins (Bellon et al. 1982); the possible physio-pathological significance of this phenomenon is unknown. The presence of deposits all along the parietal wall of vessels is compatible with the possibility of circulating immune complexes in this experimental model. Immune complexes have been shown to be present in the serum

by the Raji test and Cl_q binding test (Bellon et al. 1982). In situ formation of immune complexes is also a possibility (Van Damme et al. 1978). However, deposits are scattered in irregular clusters and their distribution seems to be more closely related to permeability than to antigenic affinity.

It is noteworthy that no inflammatory reaction was observed in these vessels. There were no polymorphonuclear leukocytes or monocytes on electron microscopy. Similar deposits in vascular walls have been observed in the chronic Bovine Serum Albumin experimental model (Brentjens et al. 1974) and in the mouse lupus-like syndrome (Acinni and Dixon 1978).

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