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Ultrastructural histochemical investigations of "dense deposit disease". Pathogenetic approach to a special type of mesangiocapillary glomerulonephritis

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Summary. Dense deposit disease is characterized by the presence of intramembranous dense deposits; their consituents are unknown but immunological and biochemical studies have demonstrated that they contain no γ-globulins or any other plasma protein. In order to clarify the nature of the dense deposits better, we investigated their most distinctive character, (marked electrondensity) by means of ultrastructural histochemistry techniques using thin sections from Formaldehyde fixed, OsO_4 postfixed and Epon embedded specimens collected for diagnostic electron microscopy. The dense deposits have a higher osmium affinity than the lamina densa of normal basement membranes, and the electrondensity is strictly osmium-dependent suggesting the presence of a lipid component. Further data, obtained using an extraction method for lipids, seems to confirm our hypothesis.

Key words: Dense deposits – Electrondensity – Type II mesangiocapillary glomerulonephritis – Ultrastructural histochemistry

Introduction

In 1962 Galle and Berger described a second type of mesangiocapillary glomerulonephritis, a disease characterized by a peculiar modification of the basement membranes (b.m.) of the kidney.

Under the electron microscope the lamina densa (l.d.) of the affected b.m. appears unusually thick and strongly electron dense, and its normal fibrillar structure seems to be masked, or replaced, by a very dense, amorphous material (Churg et al. 1979; Droz et al. 1977). These intramembranous dense deposits (d.d.) are highly specific, hence the term dense deposit disease (D.D.D.) for this glomerulopathy (Sibley and Kim 1984). The d.d. seem to be quite different from the electrondense deposits usually observed in course of immune-complex glomerulonephritides: immunoglobulins are usually absent, and the C3 fraction of complement shows a peculiar distribution along the margin, but not within the central portion of the d.d (Kim et al. 1979).

By means of chemical analysis carried on b.m. isolated from affected kidneys, Galle and Mahieu (1975) reported the presence of higher levels of sialic acid and lower levels of cystine than in normal b.m., thus suggesting a modification of the intrinsic b.m. components. However some other findings, such as the recurrence of d.d. in transplanted kidneys (Curtis et al. 1979; Droz et al. 1979; Leibowitch et al. 1979), or their lack of reactivity with specific antibodies against the basic components of normal b.m. (Fish et al. 1979; Nevins 1985), seem to be more consistent with an extrarenal origin. Further evidence is given by the demonstration of true d.d. in the splenic sinusoidal membranes of patients with D.D.D. (Ormos et al. 1979); a careful search has been carried in other organs, but was unsuccessful (Thorner and Baumal 1982).

In order to obtain a further characterization of the d.d. components we investigated the nature of their strong electrondensity by means of simple techniques of ultrastructural histochemistry. In this study we demonstrate that the electrondensity of the d.d. is due to their high osmium affinity, thus suggesting the presence of unsaturated lipids; we report also the results obtained with the application of an extraction method for lipids (Keilig 1944), which seems to support this hypothesis.

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Table 1. Histological and ultrastructural appearance of the biopsy specimens

Case #	Sex	Age	Histology and ultrastructure
1	M	17	MCGN with segmental sclerosis
2	M	13	MCGN with crescents, segmental sclerosis and endothelial proliferation
3	F	21	MCGN with crescentic formation
4	M	17	Diffuse mesangial proliferative GNF
5	F	4	Mesangial proliferative GNF with typical d.d. in mesangial areas
6	F	40	MCGN with interstitial sclerosis
7	M	18	Focal MCGN, with crescents, endothelial proliferation and capsular fibrosis
8	F	11	MCGN, with crescents, segmental sclerosis, tubular atrophy and interstitial xantomatous cells
9	F	12	MCGN with segmental necrosis, crescents and fibrin in subendothelial spaces
10	M	25	Focal MCGN with endothelial proliferation, crescents and interstitial xantoma cells
11	F	34	Focal mesangial GNF with segmental sclerosis and focal tubular atrophy; typical d.d. are seen in mesangial areas and along tubular b.m.
12	M	25	MCGN with hyalinosis, crescents, fibrin in urinary space, diffuse tubular atrophy and typical d.d. in mesangial areas
13	M	40	Lobular GNF with segmental hyalinosis and necrosis and typical d.d. in mesangial areas and along tubular b.m.

GNF: glomerulonephritis; MCGN: mesangiocapillary glomerulonephritis; d.d.: dense deposits; b.m.: basement membranes

Table 2. Electrondensity evaluation scale (1)

		Ur−Pb	H_2O_2	H_2O_2−PTA	SN	Unstained
F−OsO_4	dd	++++	0	+	++++	++
	n	++	0	+	++	0
F	dd	++/+++	n.t.	n.t.	0	0
	n	++	n.t.	n.t.	0	0
F−E−OsO_4	dd	+/++	n.t.	n.t.	+	0
	n	+/++	n.t.	n.t.	+	0

(1): 0 = electrondensity of normal basement membrane in OsO_4-postfixed, unstained sections. ++++ = electrondensity of dense deposits in OsO_4-postfixed, Ur−Pb stained sections. dd = dense deposits; *n* = normal b.m. traits; n.t. = not tested; F−OsO_4 = Formaldehyde fixed, Osmium postfixed specimens; F = Formaldehyde fixed specimens; F−E−OsO_4 = Formaldehyde fixed, lipid extracted, OsO_4 postfixed specimens; Ur−Pb = Uranyl acetate−Lead citrate; H_2O_2 = Hydrogen peroxide; PTA = Phosphotungstic acid; SN = Silver nitrate

Materials and methods

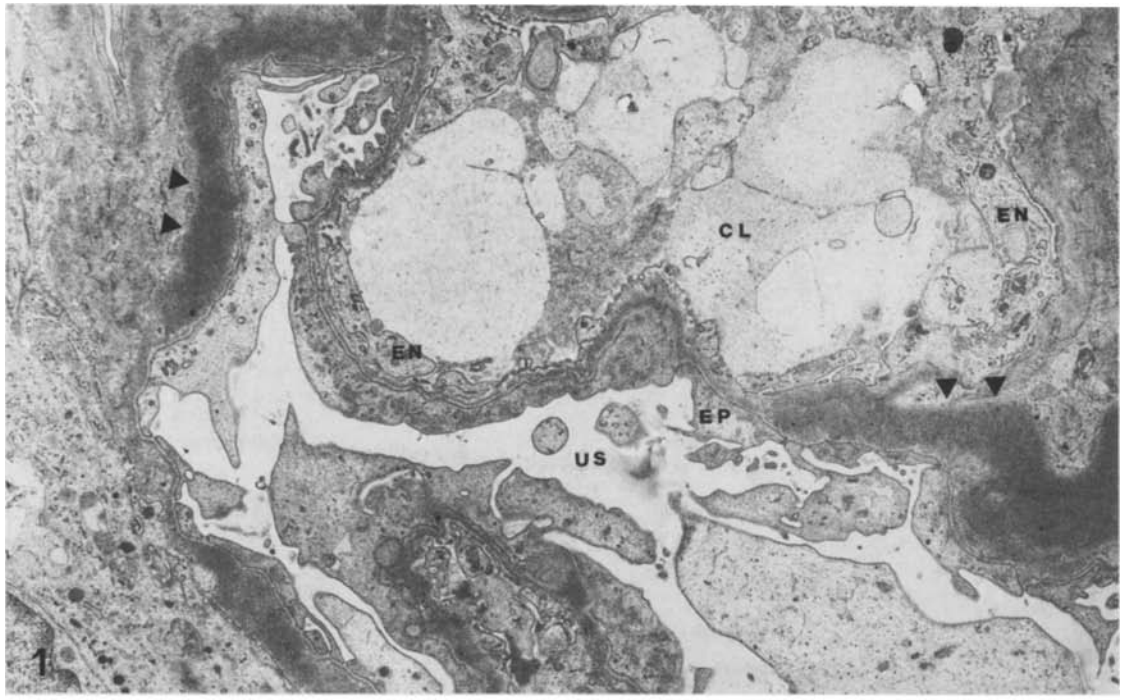
The study was performed on 13 cases which had been diagnosed as D.D.D. from a total of about 1500 renal biopsies observed during the years 1975–1986 in our Department. The histological and ultrastructural appearance of the investigated cases is summarized in Table 1.

The first step consisted of a retrospective analysis of tissue blocks already processed for diagnostic electron microscopy, i.e. fixed in 4% Formaldehyde in Phosphate buffer (0.1 M, pH 7.2) for 24 hs, postfixed in 1% OsO_4 in the same buffer for 1 h and embedded in Epon. Additionally, some fragments obtained from case n. 10 were embedded in Epon omitting OsO_4 postfixation. Thin sections obtained from the above-mentioned blocks were treated as follows:

1. Uranyl Acetate−Lead Citrate (Ur−Pb).
2. Hydrogen Peroxide (H_2O_2) 3% for 15′ at room temperature for the oxidation of reduced osmium bound to the tissue. Some of the oxided sections were also treated with Phosphotungstic acid (PTA) for the staining of glycoproteins (Faraggiana and Marinozzi 1979).
3. Silver nitrate (SN) 0.1% according to Marinozzi (1961, 1963), made by adjusting the pH of 10 cc of a 0.3% $AgNO_3$ solution to 7.5–9 with a 5% solution of borax and adding double distilled water to 30 cc, to demonstrate reduced osmium in the tissue.

Unstained sections were also observed.

The second step of our study consisted of the application of an extraction method performed on tissue fragments obtained from case n. 10. Thirty to 50 micron thick sections were cut with a S & F tissue-chopper from the Formaldehyde-fixed specimen, treated with a mixture of Chloroform-Methanol 1:1 for 18 hs. at 60 C for the extraction of all lipid components (Keilig 1944), posfixed in OsO_4 and embedded in Epon. As



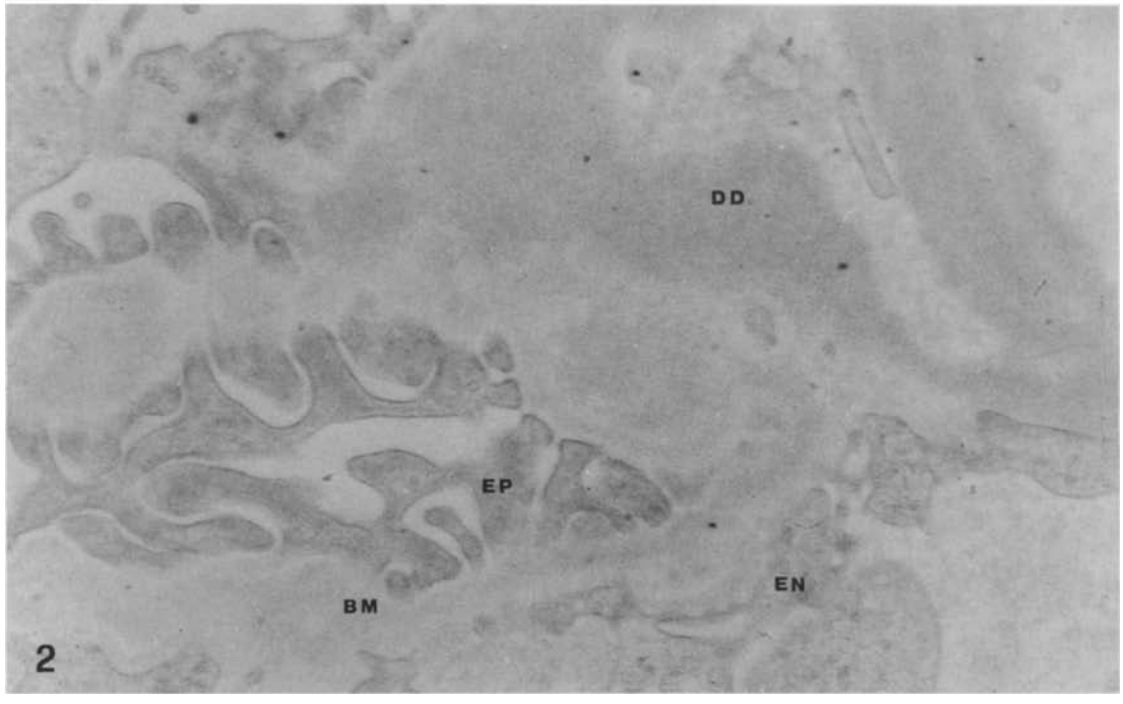


Fig. 1. In D.D.D. the lamina densa of the glomerular basement membranes is thickened and highly electron dense (Arrowheads), with abrupt transition between normal and abnormal areas. (EN = endothelial cell; EP = podocytes; US = urinary space; CL = capillary lumen). ($F-OsO_4$; Ur – Pb. × 8100)

Fig. 2. The dense deposits display their typical electron density even without treatment with Uranyl acetate and Lead citrate. (EP = podocytes; EN = endothelial cell; BM = normal basement membrane; DD = dense deposits). ($F-OsO_4$; unstained. × 22000)

controls, some 50 micron sections obtained from the same specimen were placed for 18 hs. at 60 C in the buffer solution. The extraction procedure was also applied to normal tissue obtained from surgical specimens of human kidneys explanted for carcinoma.

Thin sections obtained from the above-mentioned specimens were stained as already described.

Electron microscopy was performed on a Siemens 102 instrument, with an acceleration voltage of 80 Kv and with an objective aperture of 50 microns. As variables, the electronden-

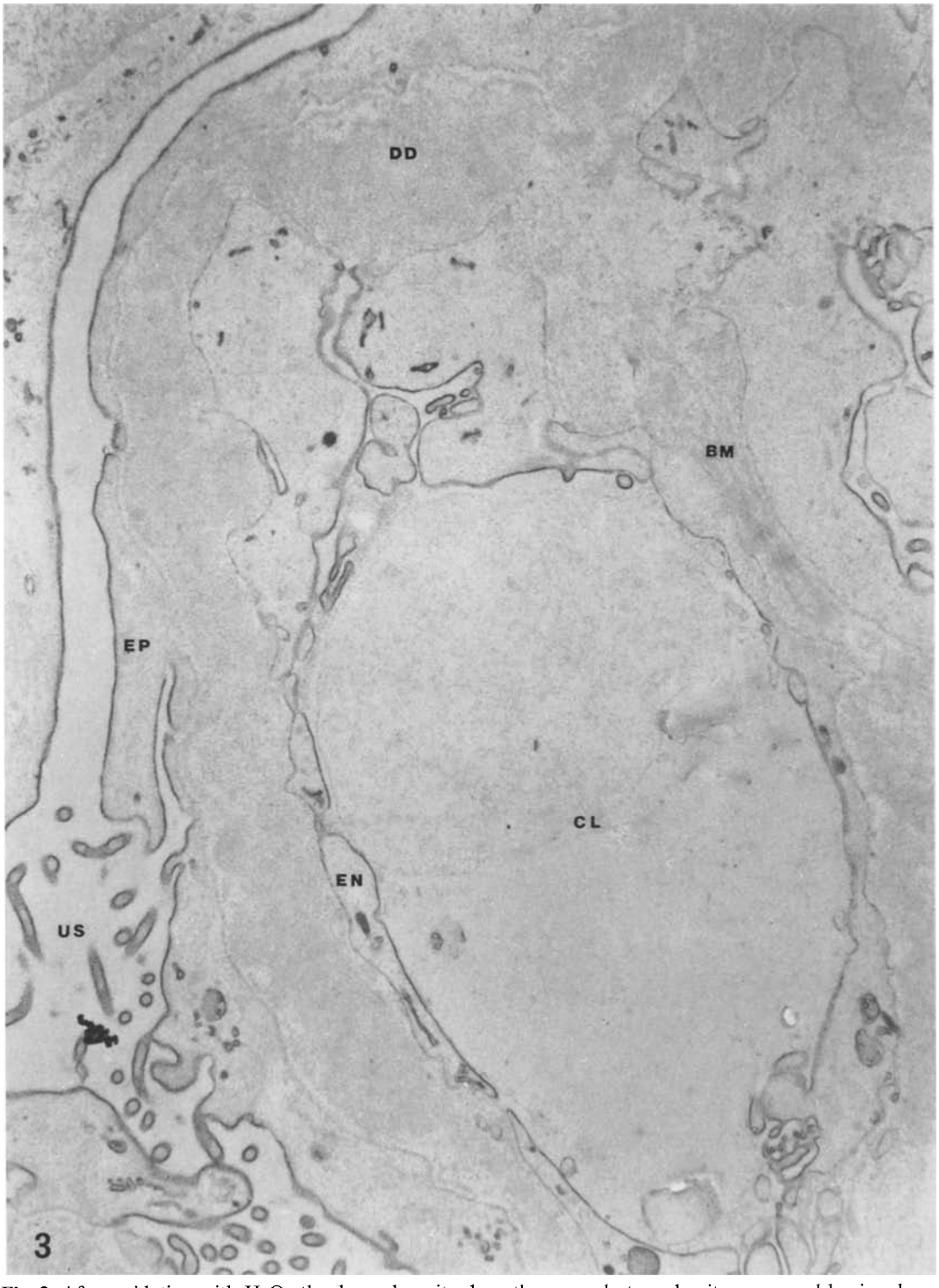


Fig. 3. After oxidation with H_2O_2 the dense deposits show the same electron density as normal lamina densa. Further treatment with PTA highlights the cell coat of podocytes and endothelial cells. (EP = podocytes; EN = endothelial cell; CL = capillary lumen; US = urinary space; BM = normal basement membrane; DD = dense deposits). ($F-OsO_4$; H_2O_2 – PTA. × 21 500)

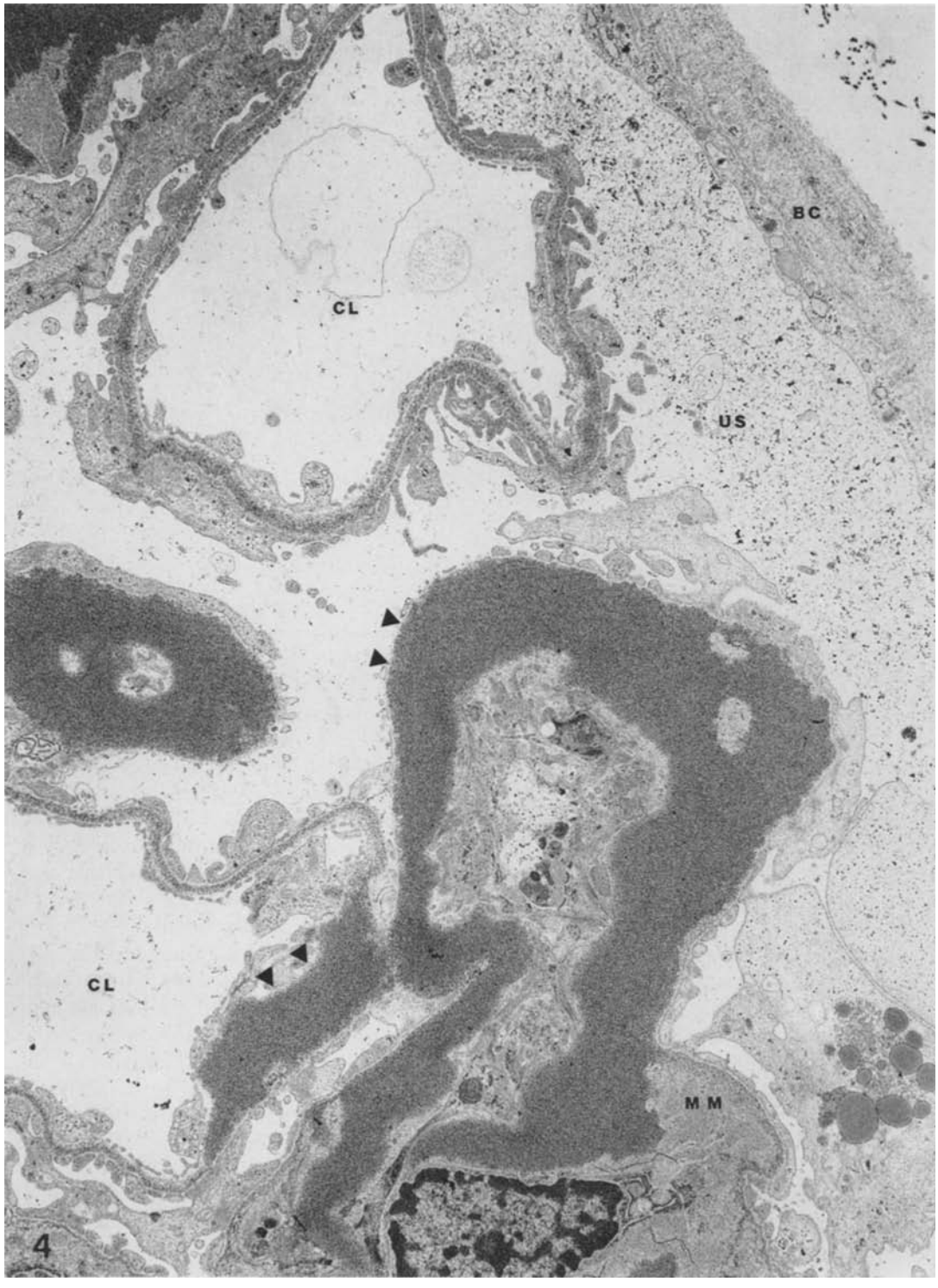


Fig. 4. Unlike normal capsular and capillary basement membranes and mesangial matrix, the dense deposits strongly react with silver nitrate (*arrowheads*), with the deposition of large amounts of Ag grains. (CL = capillary lumen; US = urinary space; BC = Bowman's capsule; MM = mesangial matrix). ($F-OsO_4$; SN. ×8100)

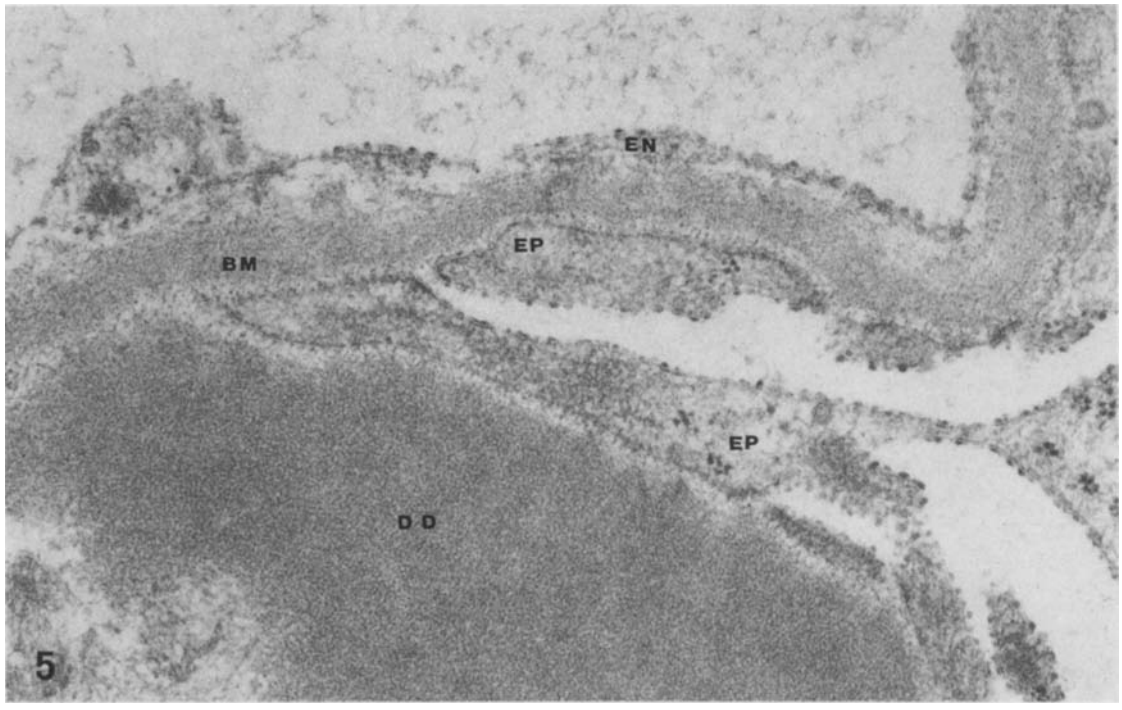


Fig. 5. When OsO_4 postfixation is omitted, the dense deposits show a compact and homogeneous structure; the difference in electrondensity they usually displayed between normal and altered traits in routine, OsO_4 postfixed preparations, is reduced. (EP = podocytes; EN = endothelial cell; BM = normal basement membrane; DD = dense deposits). (F; Ur – Pb. × 23 500)

sity of the d.d. and of the normal b.m. were evaluated indipendently by the authors using a semiquantitative scale (see Table 2).

Results

On specimens postfixed with OsO_4 thin sections treated with Ur-Pb showed the altered b.m. as already described in the literature (Fig. 1). The l.d. was unusually thickened and highly electron dense, with a more homogeneous and compact structure than normal. The d.d. were readily recognized even in unstained sections (Fig. 2), and they contrasted strikingly with the low electrondensity of the unaffected portions of the b.m. After treatment with H_2O_2, all osmiophilic components of the tissue lost their electrondensity almost completely; in particular the d.d. showed no difference, in contrast with the normal l.d.

Further treatment of the oxided sections with PTA gave excellent staining of the glycoproteins of the podocytes cell coat, with only a slight contrast enhancement of the l.d. of the b.m. (Fig. 3). However, the electrondensity of the d.d. was always lower than that observed in unoxided, unstained sections.

Treatment of the thin sections with SN produced a large number of Ag grains on the altered areas of the b.m. (Fig. 4).

On specimens fixed with formaldehyde only the d.d. displayed their typical homogeneous and compact structure after staining with Ur – Pb. This was readily differentiable from the normal l.d. (Fig. 5). Unstained sections showed a total absence of the typical electron density of the d.d., with no difference in contrast between altered and normal b.m.

Thin sections treated with SN showed a diffuse lack of contrast of all structures: no silver grains were observed on both altered or normal b.m.

Thin sections, obtained from tissue blocks processed according to the method of Keilig before OsO_4 postfixation and stained with Ur – Pb, showed a general decrease in the electron density of all the osmiophilic structures; the affected tracts of the b.m. displayed the same contrast of the normal l.d. (Fig. 6). This was confirmed by observation of the unstained sections (Fig. 7).

SN stain again resulted in low contrast of all tissue components; the d.d. exhibited the same electron density as the unaffected areas of the b.m. (Fig. 8).

The control blocks, in which buffer solution had been used instead of the Chloroform-Metha-

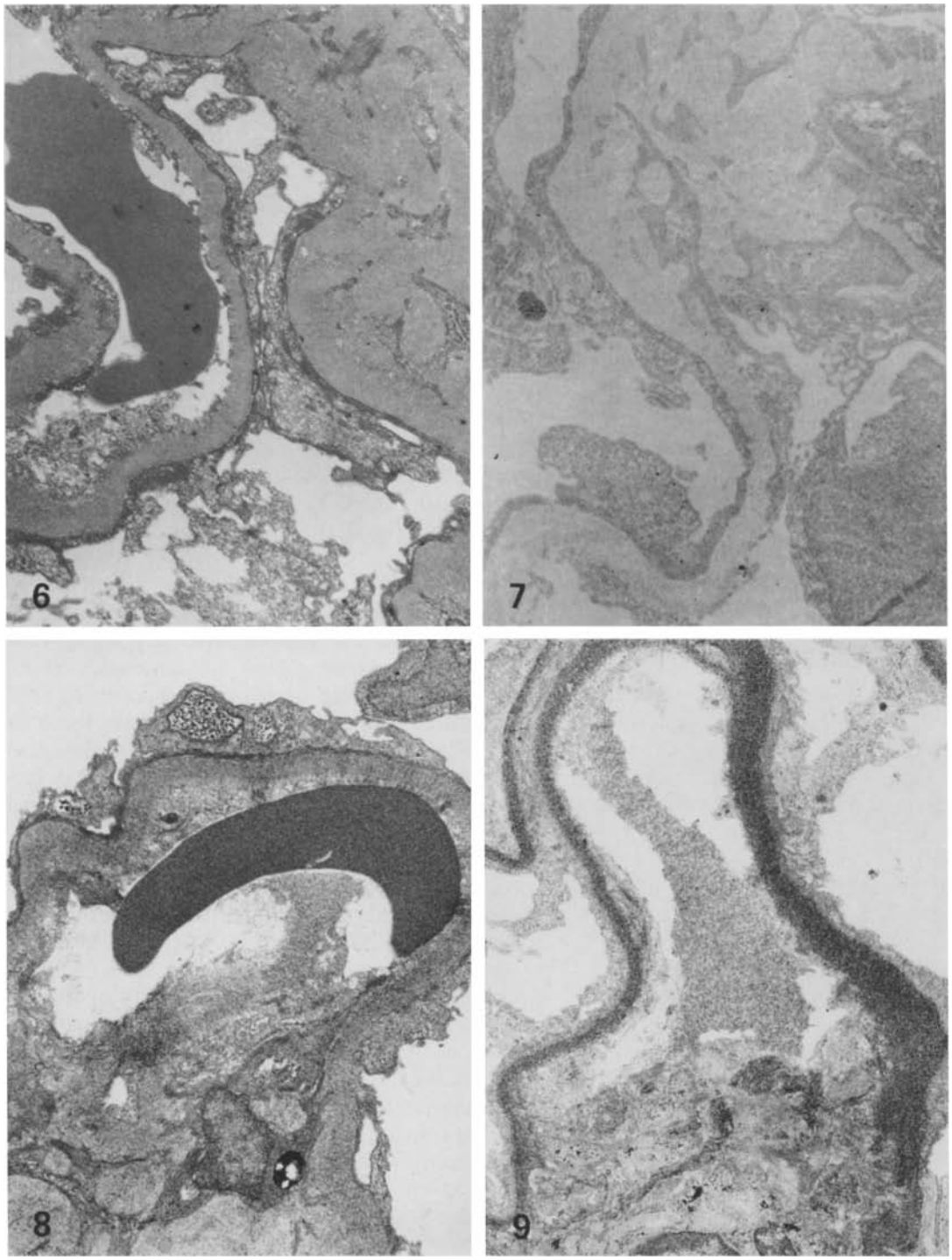


Fig. 6–9. Lipid extraction with Chloroform – Methanol (C/M). In the treated specimens the altered traits of the glomerular basement membranes display the same contrast of normal lamina densa and mesangial matrix (Fig. 6; F–C/M–OsO_4, Ur–Pb. ×12500); the low electrondensity of the dense deposits is best appreciated when Ur–Pb is omitted. (Fig. 7; F–C/M–OsO_4, unstained. ×16000). Silver nitrate treatment on thin sections from the same tissue blocks shows a low reactivity of the affected basement membranes. A loose, finely fibrillar structure is also evident. (Fig. 8; F–C/M–OsO_4, SN. ×12500). Silver nitrate treatment on thin sections from control blocks gives a strong reactivity of the dense deposits, as in routinely processed specimens. (Fig. 9; F-Buffer–OsO_4, SN. ×7500)

nol mixture, did not show substantial differences from the routinely processed tissue fragments (Fig. 9). Lastly, no relevant variations in electron-density of the b.m. were observed in the normal tissue specimens treated with the extractive procedure.

Discussion

D.D.D. is characterized by the presence of peculiar intramembranous deposits along the b.m. of the kidney. Some other signs, such as a great variability of the histological appearance (Sibley and Kim 1984), the total absence of immunoglobulins in the d.d. (Fish et al. 1979; Nevins 1985), the almost invariable evidence of hypocomplementaemia, the presence of C3NeF in the serum with activation of the C3 fraction of complement through the alternative pathway (Kim et al. 1979; Leibowitch et al. 1979; Cameron et al. 1970), and the constant recurrence of the lesions in transplanted kidneys (Curtis et al. 1979), differentiate the disease from other forms of mesangiocapillary glomerulonephritis.

Previous works have shown that the formation of the d.d. is the first step in the pathogenesis of the disease: full-blown d.d. were easily detected quite early in the transplanted kidneys, in the absence of other clinical and histological signs of the disease (Curtis et al. 1979; Droz et al. 1979; Leibowitch et al. 1979).

To date the exact nature of the d.d. is still unknown, though several attempts have been made in order to obtain a better histological characterization (PAS, PASM, Thioflavine T, etc.) (Churg et al. 1979; Date et al. 1982). They are identified with certainty only under the electron microscope, on the basis of their peculiar, high electron density. It is generally admitted that electron density of biological substances may be due to two different mechanisms: it can be either a specific characteristic of the biological material, electron dense "per se", or be the result of the fixing and/or staining procedures performed on the tissues.

Our data, obtained by the oxidation of thin sections of OsO_4-postfixed tissue blocks to remove reduced osmium bound to the tissue (with or without further treatment with PTA), or with staining with SN to enhance the electron density of osmiophilic sites (Marinozzi 1961; Marinozzi 1963), have demonstrated that the typical electron density of the d.d. is due to their strong osmium affinity, much higher than the l.d. of the normal b.m. The lack of relevant staining differences between normal l.d. and the d.d. after H_2O_2 – PTA treatment suggests that there are no substantial variations in amount of the b.m. glycoproteins rich in –OH residues (Marinozzi 1968; Rambourg 1968).

Confirmation of our results comes from the observation of thin sections obtained from tissue blocks in which OsO_4 postfixation was omitted: in unstained sections the d.d. showed no significant differences from normal b.m., when compared on the basis of electron density alone.

Among all biological substances, unsaturated, double-bond rich fatty acids are the more prone to react with OsO_4, with formation of reduced osmium in the tissue and consequent increased electron density of the reactive structures (Bahr 1954; Stoeckenius 1957; Stoeckenius and Mahr 1965). The application of Keilig's procedure, which is known to extract all lipid components, before OsO_4 postfixation, demonstrated a dramatic decrease in electrondensity of the d.d.

Our results therefore suggest the presence in the d.d. of a highly osmiophilic component, extractable with lipid solvents. This is likely to be a compound rich in unsaturated fatty acids. This viewpoint is in keeping with the demonstrated association of the disease with other diseases of lipid metabolism, such as partial lipodystrophy (Sissons et al. 1976). The well-known activation of the complement through the alternative pathway by bacteria with a glycolipid-rich capsule (Muller-Eberhard 1975) is also of interest.

The presence of a lipid component should be confirmed by biochemical analysis on isolated basement membranes. Unfortunately our study was performed on small tissue fragments obtained by percutaneous needle biopsy carried out for diagnostic purposes, hence the impossibility of collecting material for biochemical investigations.

In conclusion, the presence of high levels of a lipid in the d.d. provides additional evidence that the formation of d.d. occurs as a result of the deposition of substances of extrarenal origin. The renal involvement would be considered as a localized manifestation of a systemic disease, probably of metabolic origin.

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