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Detection of leptospiral antigen (*L. interrogans* serovar *copenhageni* serogroup Icterohaemorrhagiae) by immunoelectron microscopy in the liver and kidney of experimentally infected guinea-pigs

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Summary. Guinea-pigs were experimentally infected with *L. interrogans* serovar *copenhageni* serogroup Icterohaemorrhagiae and their liver and kidney were studied by immunoelectron microscopy using the post embedding indirect immunogold labelling technique. Primary antibody was a purified rabbit anti-serum produced against the same leptospiral strain used in the inoculum. Gold-labelled leptospiral antigen (LAg) was found close to cell membranes of hepatocytes, kidney tubular cells and endothelial cells of the interstitial capillaries of the kidney. Afterwards it was internalized by hepatic and tubular cells, and eventually found in lysosomes. Phagolysosomes of Kupffer cells were also found to contain remnants of degraded leptospires and gold-labelled LAg. Gold-labelled intact leptospires were detected at the enlarged intercellular spaces between hepatocytes at the areas of hepatic cell plate disarray, showing the potential for leptospiral migration during the septicaemic phase of the disease potentially contributing to the pathogenesis of the lesions.

The affinity of leptospiral antigenic material for cell membranes suggests an initial interaction with cell surface proteins followed by its internalization and cell damage. The nature of antigenic material detected, however, remains undefined; it may be a toxin, an enzyme or any other factor/s involved in leptospiral virulence.

Keywords: leptospiral antigen, *L. interrogans* serovar *copenhageni* serogroup Icterohaemorrhagiae, immunoelectron microscopy, liver, kidney, guinea-pig

Leptospirosis, a world-wide infection of zoonotic origin is caused by spirochetes of the *Leptospira interrogans* complex and occurs in Brazil in endemic and epidemic bouts, chiefly during the rainy season. The most common leptospire which affects man and is usually the cause of the Weil's syndrome is *Leptospira interrogans* serogroup Icterohaemorrhagiae. Guinea-pigs inoculated with a virulent strain of this serogroup show severe hepato-renal disturbances similar to the Weil's syndrome described in humans and this provides an adequate model to study the morphological events of the disease.

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The pathogenesis of human and animal leptospirosis is poorly understood but includes migration of leptospires and/or virulence factors which may or may not be antigenic. Previous work (Ellis *et al*, 1983; Alves *et al*, 1987) has shown that immunohistochemical methods are useful for the demonstration of leptospires and/or their antigenic products in tissues of humans and experimentally infected animals. However, they have not been identified at an ultrastructural level by immunoelectron microscopy techniques, and this is the purpose of the present study.

Material and methods

The strain 14/89 of *L. interrogans* serovar *copenhageni* serogroup Icterohaemorrhagiae, isolated from a patient dying of Weil's syndrome, and grown in Fletcher's medium, was used in this experiment. A preliminary test experiment, inoculating guinea-pigs of random sex weighing 150–180g with this culture, was run in order to establish virulence and to maintain a fairly constant course for the disease. When signs of the disease, such as jaundice, became evident, blood obtained by cardiac puncture was cultured. Subsequently 10 guinea-pigs of random sex were inoculated by the intraperitoneal route with 1 ml of the 7-day cultures at 28°C in semi-solid Fletcher medium (DIFCO) enriched with 10% normal rabbit serum (10^7–10^8 leptospires), using the blood obtained from the previously infected guinea-pigs. Two test groups were defined, one of animals sacrificed at the 3rd–4th day after inoculation, when previous studies did not find significant lesions, and the other of animals sacrificed at the 5th–6th day, when signs of the disease were evident and pathological findings prominent. Immediately before sacrifice, blood obtained by cardiac puncture was cultured and confirmed positive for leptospires in both groups. Three non-inoculated (normal) guinea-pigs were used as controls.

Necropsies were performed immediately after death and liver and kidney fragments were fixed in Bouin's fluid, embedded routinely in paraffin, cut 4–5 μm thick and used for conventional histology and immunohistochemistry (Alves *et al*, 1987).

For immunoelectron microscopy, fragments of liver and kidney were initially fixed in a mixture of 4% of paraformaldehyde and 0.2% glutaraldehyde in phosphate buffer 0.1 M pH 7.3 with 2.5% sucrose. In three animals the fragments were transferred to a 0.3% solution of osmium tetroxide in phosphate buffer 0.1 M pH 7.3 for 1 hour at 4°C. Afterwards both the osmium tetroxide and non-osmium tetroxide fixed fragments were transferred to a solution of 8% paraformaldehyde in Hepes 200 mM and embedded in hard-grade LR White resin at 53°C.

Semithin sections stained by 1% methyl-

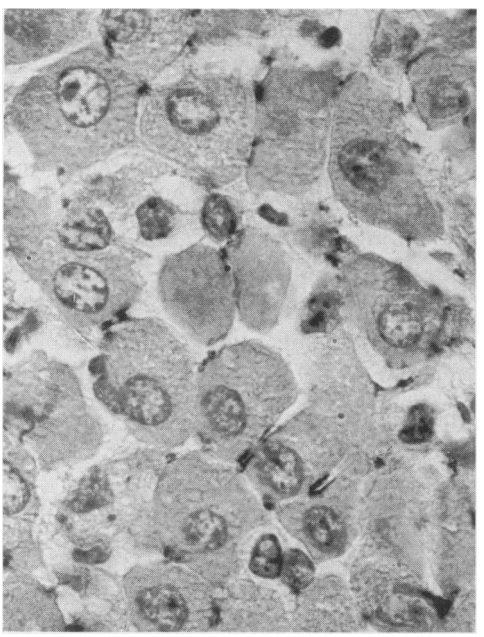

Fig. 1. Guinea-pig liver at the late phase of the leptospiral infection. Dark LAg deposits are seen on the hepatocyte cell membrane. There are areas of cell plate disarray and hepatic cells necrosis. Immunohistochemistry (PAP) counterstained with haematoxylin. × 500.

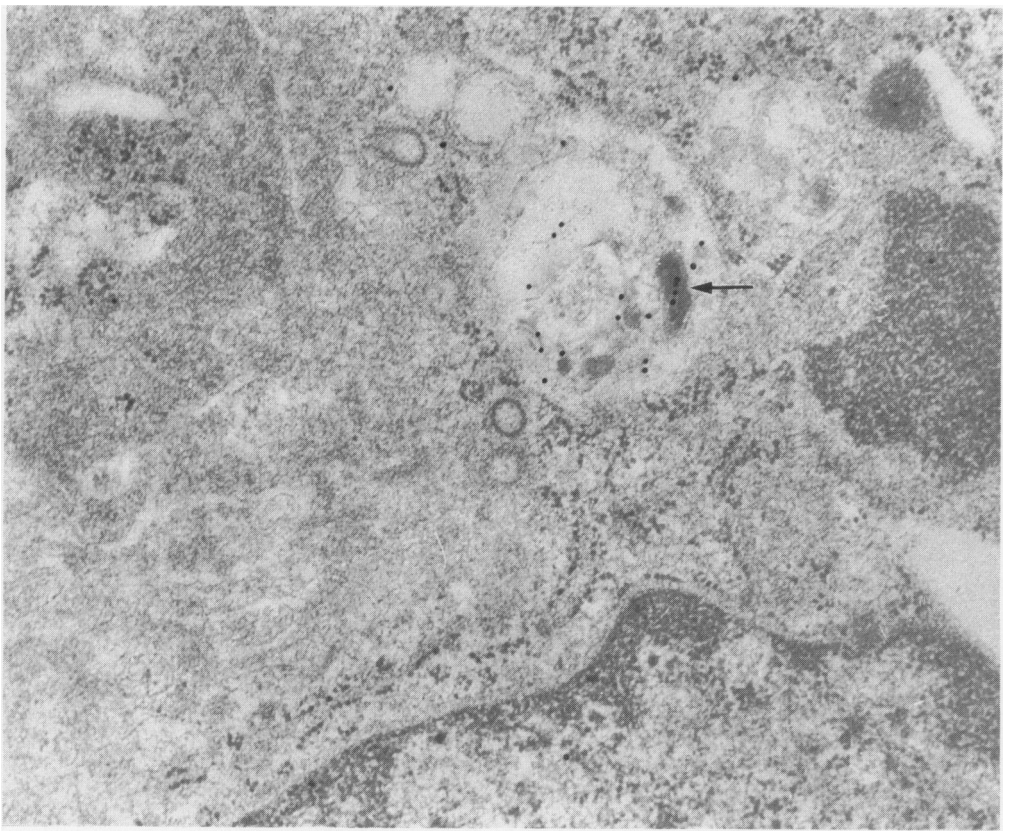

Fig. 2. Cytoplasm of activated Kupffer cell showing phagolysosome gold-labelled with anti-LAg serum. A remnant of leptospire is also seen as an elongated dark structure (arrow). Immunoelectron microscopy. ×45 000.

ene blue and azure II were used to select representative areas. Ultrathin sections were examined in a Zeiss EM 9S electron microscope. The post embedding indirect immunogold technique was performed essentially as described by Roth (1983) and Roth and Heitz (1989). The nickel grid-mounted thin sections were sequentially incubated with (a) saturated solution of sodium metaperiodate for 30 minutes; (b) TRIS-buffered saline (TBS), pH 7.4, for 15 minutes; (c) 1% bovine serum albumin (BSA), Triton X-100 0.025% and Tween 20, 0.025% in TBS solution 0.02 M pH 7.4. (d) The primary antibody, rabbit anti-serum to leptospires was produced using the same strain as for inoculation of the guinea-pigs. Adult rabbits were immunized with sequential i.v. doses of 1,2,4 and 4ml suspensions of organisms with one week between doses; the animals were bled 7 days after the last inoculation. The microscopic agglutination test revealed a 1:6400 titre. The IgG obtained by sodium sulphate fractionation, dialysed and purified in Sephadex G100 and DEAE cellulose, was used in a 1:100 dilution in TBS with 1% BSA, Triton and Tween 0.025% each, overnight at 4°C (e). The grids were washed overnight with TBS, Triton X-100 and Tween 20, 0.025–0.05% each (f). BSA as described above. (g),

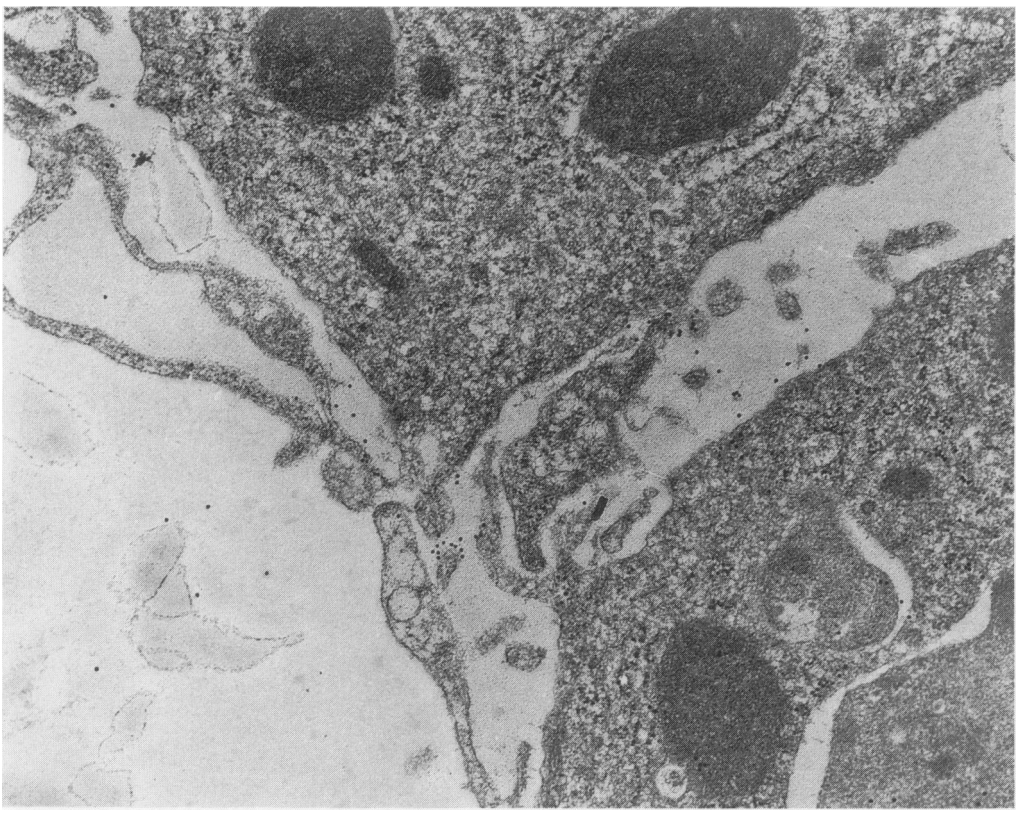

Fig. 3. LAg gold-labelled deposits on hepatocyte microvilli, perisinusoidal space and the widened intercellular spaces between hepatic cells at zones of hepatic cell plate disarray. Endothelial cell cytoplasm is swollen and microvilli are focally absent. Immunoelectron microscopy. × 40 000.

Protein A Gold, 15 nm (E.Y. Laboratories, Inc., San Mateo, California, USA), diluted 1:50 in TBS with 1% BSA, Triton X-100 0.025% and Tween 20, 0.025% for 60 minutes in a moist chamber at room temperature (h). The grids were washed as described in (e). Finally, grids were rinsed with distilled water and sections were contrasted with uranyl acetate and lead acetate.

Negative controls included: (a) liver and kidney of normal (healthy) guinea-pigs; (b) replacement of primary antibody by normal rabbit immunoglobulin; (c) replacement of primary antibody by the same serum after absorption with the strain used as inoculum.

Results

Liver

Previous studies in this experimental model have failed to show liver lesions by light microscopy in the first test group (3rd–4th day after inoculation) (De Brito *et al*, 1966; Alves 1988). Except for occasional deposits in Kupffer cells, no leptospiral antigen (LAg) was detected by immunohistochemical techniques in this group or in controls.

Ultrastructural studies performed in LR White embedded thin sections, showed an essentially normal pattern of hepatocytes, portal triads and centrolobular vein. How-

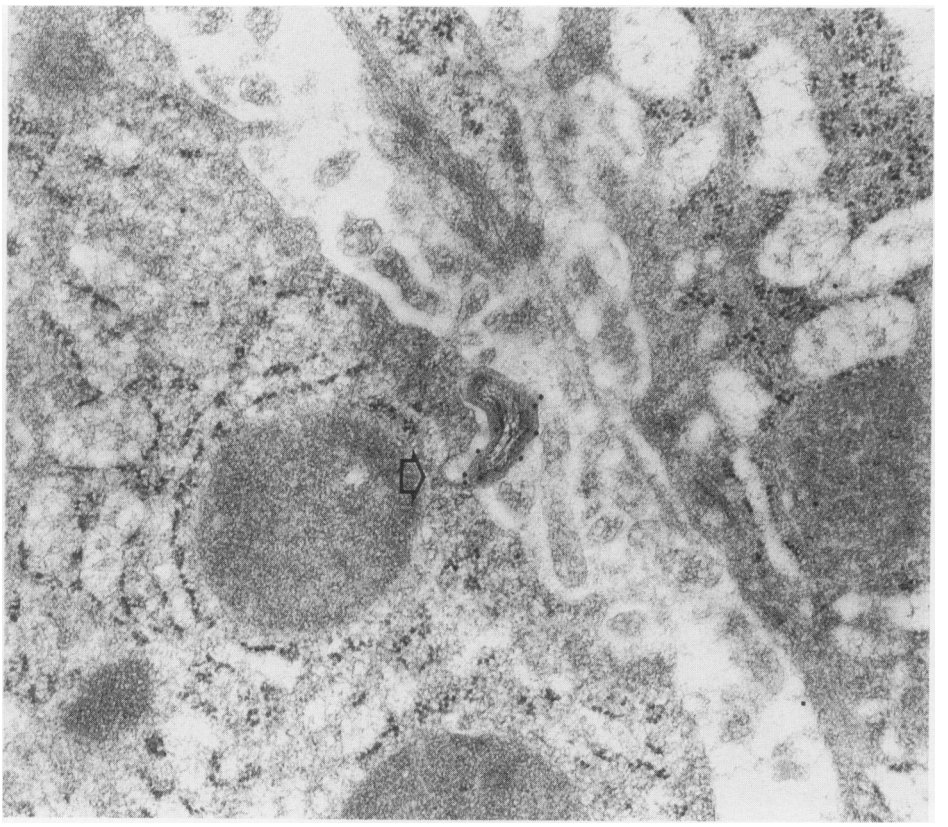

Fig. 4. Widened intercellular space between hepatic cells permeated by a gold-labelled leptospire. An early endocytic vesicle is being formed close to the leptospire at the hepatic cell membrane (open arrow). Immunoelectron microscopy. ×45 000.

ever, in one animal positive immunogold labelling for LAg was detected close to the cell junction of a bile ductule.

The second test group (5th–6th day after inoculation) showed morphological findings as previously described by De Brito *et al.* (1966) and Alves (1988).

Immunohistochemistry showed LAg deposits in the portal interstitium and also phagocytosed by enlarged Kupffer cells. LAg deposits showed a close association with liver-cell membranes and a marked concentration in areas of trabecular disarray (Fig. 1).

Ultrastructural findings were essentially as previously described (De Brito *et al*, 1966). Immunoelectron microscopy showed swollen endothelial cells, activated Kupffer cells, with many phagolysomes occasionally gold labelled with anti-LAg serum (Fig. 2). Positive gold labelling was occasionally observed over remnants of phagocytosed leptospires (Fig. 2). The perisinusoidal space was enlarged, containing cell debris, and the hepatic cells showed loss of microvilli. The immunogold method revealed focal labelling on altered microvilli and in the perisinusoidal space (Fig. 3). Intercellular spaces between hepatic cells were widened and again LAg was gold labelled on the villi with

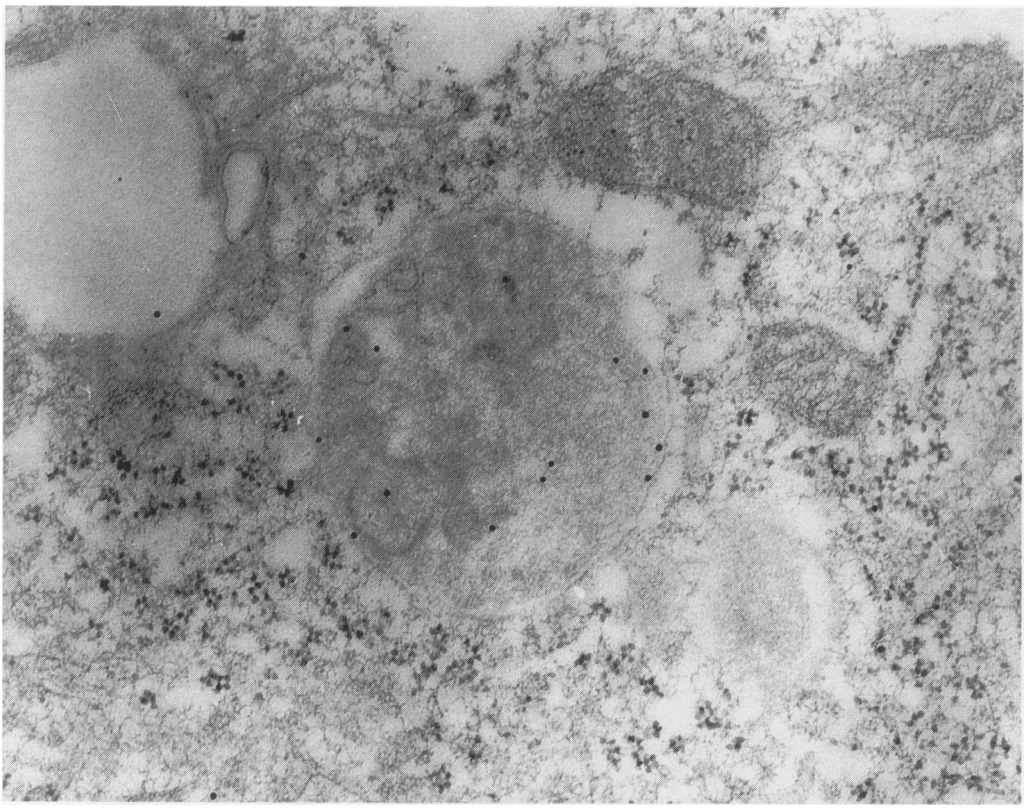

Fig. 5. Hepatic cell lysosome containing LAg gold-labelled deposits. Immunoelectron microscopy. ×45000.

an occasional intact leptospire permeating the space (Fig. 4). Infrequently gold-labelled LAg was detected in lysosomes in liver cells (Fig. 5).

Kidney

Except for interstitial oedema, no significant kidney lesions were observed by light and electron microscopy and no leptospiral antigen was detected by immunohistochemical techniques in the first test group or in the controls. However, immunoelectron microscopy disclosed gold-labelled LAg at the base of a proximal tubule cell in one animal.

In the second test group, light and ultrastructural findings were as previously described (De Brito *et al.* 1966; Arriaga *et al.* 1982; Alves *et al.* 1991).

Immunohistochemistry showed focal LAg deposits in the kidney interstitium both in the cortex and the medulla. LAg was found around tubules, rarely crossing their walls, or in small amounts adhered to the luminal border, between tubular cells or free in the tubular lumen (Fig. 6). There was not a predominance of the deposits in any particular segment of the nephron.

Immunoelectron microscopy detected considerably less LAg in the kidney compared to the liver. Immunogold methods revealed focal gold-labelled deposits of LAg

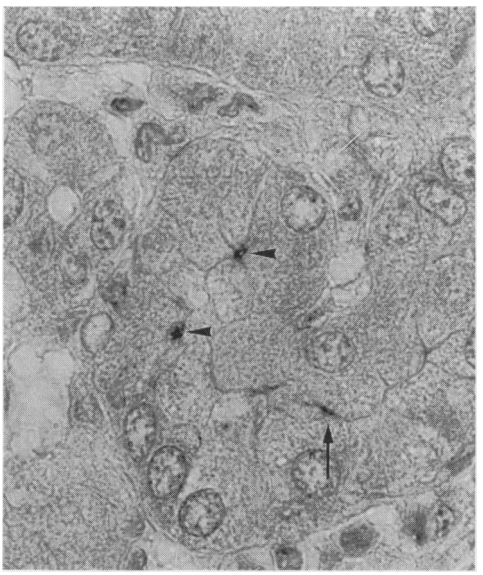

Fig. 6. Dark LAg deposits adhered to the luminal border (arrow heads) and between (arrow) enlarged tubular cells of the guinea-pig kidney at the late phase of the infection. Immunohistochemistry, counterstained with haematoxylin. × 500.

on the brush border of proximal tubular cells (Fig. 7). Labelling of tubular cell lysosomes with anti-LAg serum was occasionally seen and gold labelling was also detected over cell membranes delimiting enlarged basolateral infoldings of tubular cells. Also, gold-labelled antigen deposits were detected in the cytoplasm of swollen endothelial cells, close to the cell membrane (Fig. 8).

Discussion

Leptospirosis may be regarded as a generalized, systemic illness rather than a disease of a specific organ or tissue. Pathogenic leptospires, after penetrating the barriers of the host, invade the blood stream and spread throughout the body, affecting many organs and thereby producing the protean manifestations of the disease (reviewed by Feigin & Anderson 1975). Prominent in this septicaemic picture, however, are liver and kidney manifestations, as well as vascular injury (De Brito *et al*, 1979). During the septicaemic phase, bacterial migration (Sitprija *et al*, 1980), toxin/s, enzymes and/or antigenic products liberated by bacterial lysis (Yasuda *et al*, 1986) might account not only for the increased vascular permeability which might be regarded as the earliest manifestation of the disease, but also for alterations of cell cohesion which culminate in hepatic cell plate disruption seen in the late phase of both human and experimental disease. Tissue damage and haemoglobinuria are often associated with leptospirosis and cytotoxins and haemolysins, which have been demonstrated both in the culture supernatant and in leptospires, are involved in their pathogenesis (Segers, 1991).

Attachment of pathogenic leptospires to host mammalian cells or tissues may be interpreted as an important initial step in the establishment of leptospiral infection. Ballard *et al.* (1986) showed that virulent strains of serovar *copenhageni* and *ballum* attached themselves to epithelial cells in culture within 3 hours of infection whereas an avirulent variant did not adhere to these cells at all. Furthermore, pathogenic leptospires can penetrate tight-junctioned epithelial and endothelial cell monolayers and can be seen also within host cells either in cell vesicles or free in the cytoplasm (Thomas & Higbie 1990). Ito and Yanagawa (1987) demonstrated that highly virulent lines of *Leptospira interrogans* serovar *copenhageni* attached to extracellular matrix of mouse fibroblast more effectively than avirulent lines of the same strains, suggesting again a relationship between attachment and virulence.

As previously reported, Alves (1988) and Alves *et al*, (1991) found a remarkable affinity of LAg for kidney tubular cells and hepatocytic membranes, frequently associated with swollen or condensed cells which were losing cell cohesion. Spiral elongated forms of LAg, when detected, were suggestive of intact leptospires, which were shown

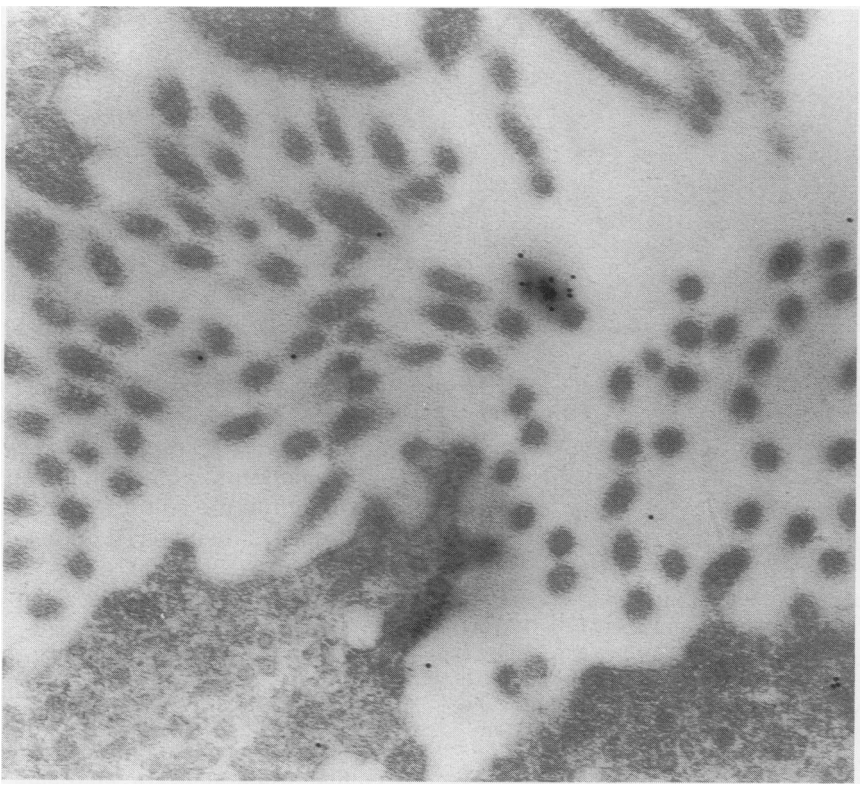

Fig. 7. LAg gold-labelled deposits on the brush border of proximal tubule cells. Immunoelectron microscopy. ×45 000.

to proliferate at the early phase of the experimental infection.

Immunoelectron microscopy confirmed and extended these studies. Gold labelling of LAg was seen, as was expected, on intact leptospires which permeate chiefly between detached hepatic cells showing the potential importance of leptospire migration in the pathogenesis of the disease. They can move in between the cells by detaching the tight junctions between the cells (De Brito *et al*, 1966; Segers 1991). However, gold labelling was also detected close to cellular membranes of hepatocytes, on the brush border of tubular cells and in endothelial cells. The role of the mononuclear phagocytic system in the clearance of leptospires and/or leptospiral antigens was demonstrated when gold labelling of phagolysosomes in Kupffer cells was observed, occasionally with remnants of leptospires present. Perhaps the role of hepatic cells in the clearance mechanism of LAg is secondary to initial clearance and modification by Kupffer cells, as has been shown for Gram negative infections (Fox *et al.* 1990).

Gold labelling of lysosomes in hepatic and kidney tubular cells suggests that these cells internalize LAg, probably by a non-specific adsorptive pinocytosis; after passing through the endocytic pathway LAg is eventually found in lysosomes.

It is not certain whether the gold-labelled antigenic material close to cell membranes is causing damage to the cell or is degraded

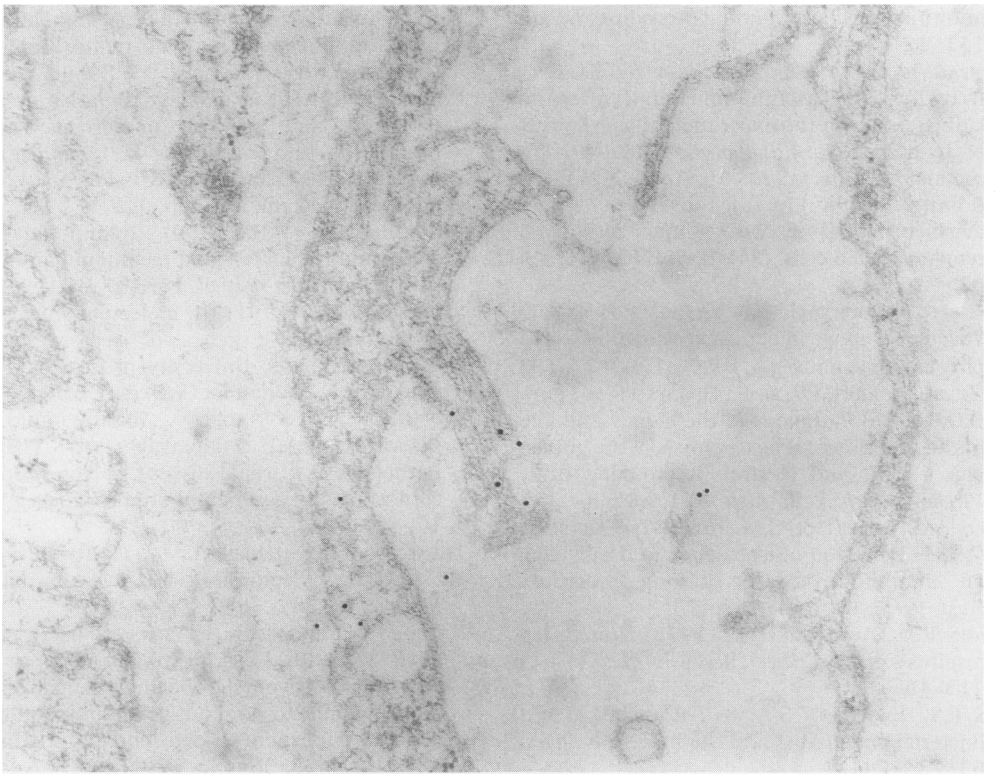

Fig. 8. LAg gold-labelled deposits in the cytoplasm of swollen endothelial cells of the kidney interstitium. Immunoelectron microscopy. × 50 000.

migrating leptospires. Its presence, however, in contact with damaged hepatocytes, kidney tubular and endothelial cells of interstitial capillaries suggests an initial interaction with cell surface proteins followed by internalization and cell damage. Its precise nature, however, remains undefined; it may be a toxin, an enzyme or any other antigenic factor involved in leptospiral virulence. However, the possible role played by substances other than those shown by immunohistochemical and immunoelectron microscopy techniques cannot be discarded in the pathogenesis of the tissue damage in leptospirosis.

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