Electron Microscopy and Immunoelectronmicroscopy of Cytoplasmic Hepatitis B Antigen in Hepatocytes

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The localization of hepatitis B antigen (HB Ag) and the nature of the virus-like particles in hepatocytes of patients with HB antigenemia are controversial. In many reports, numerous virus-like particles have been demonstrated in hepatocytic nuclei; the few reported in the cytoplasm are insufficient in number to explain the intense cytoplasmic fluorescence after staining with fluoresceinated antibody to HB Ag (HB Ab). We found numerous tubular and circular structures, measuring 20 to 30 nm in diameter, in the cisternae of the excess smooth endoplasmic reticulum (ER) of varying numbers of hepatocytes in 13 of 16 HB Ag carriers and in 4 of 9 patients with HB Ag-positive chronic hepatitis corresponding to cytoplasmic HB Ag-specific fluorescence. Direct immunoelectronmicroscopy using peroxidase-labeled HB Ab revealed that the intracisternal bodies and the surrounding membranes contain HB antigenic determinants. These bodies are an ultrastructural correlate of cytoplasmic HB Ag. It is suggested that they are virally coded coat material rather than the mature hepatitis B virus or its core (Am J Pathol 75:489–502, 1974).

A VARIETY OF PARTICLES has been reported to be present in the serum or liver of patients with hepatitis B antigenemia (hepatitis B antigen, HB Ag). The three different structures in the serum (*ie*, 20-nm spheres; tubules, also 20 nm in diameter and varying in length;[1] and 42-nm Dane particles, with an outer coat and a 27-nm electron-dense inner core[2]) seem to have at least one antigen in common, since all three morphologic forms take part in the formation of immune complexes after addition of antibody to HB Ag (HB Ab).[3,4] In hepatocytes, 20- to 25-nm spherical particles have been demonstrated by many investigators[5] in the nuclei and probably correspond to 27-nm noncoated spherical particles in the negatively stained preparations of liver homogenates which are ultrastructurally identical to the core of the Dane particles.[6–8] On direct and indirect immunoelectronmicroscopy, the intranuclear particles were shown to react with Hb Ab.[9,10] These

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particles seem to be antigenically similar to the core of the Dane particles, but antigenically different from the spheres, tubules and coat of the Dane particles,[11] since some antibodies react with the core and others with the spheres, tubules and coat of the Dane particles.[12,13] Similarly, some fluoresceinated antibodies to Hb Ag react with hepatocytic nuclei, others with the hepatocytic cytoplasm or both when incubated with livers from patients with HB antigenemia.[14,15] This suggests that the intranuclear particles are antigenically different from cytoplasmic HB Ag. The ultrastructural correlate of the nuclear fluorescence appears to be the 20- to 25-nm spherical particle in the hepatocytic nuclei.[5] Cytoplasmic virus-like particles have been observed less frequently and usually not in sufficient number to explain the intense cytoplasmic fluorescence of the hepatocytes of some patients with HB antigenemia.[16] In a previous study based on light, fluorescent and electron microscopic investigations, we detected numerous 20- to 30-nm tubular and circular structures in the cisternae of the endoplasmic reticulum (ER) of the hepatocytic cytoplasm of 14 persons with HB antigenemia corresponding to intense cytoplasmic fluorescence after staining of liver sections with fluoresceinated HB Ab.[17] Preliminary indirect immunoelectronmicroscopic studies using ferritin-labeled antibodies indicated that the tubular and circular structures as well as the surrounding membrane of ER reacted with HB Ab.[17] To learn more about cytoplasmic HB Ag, these studies were extended by investigation of a larger material, by a detailed ultrastructural examination of this material and by application of direct immunoelectronmicroscopy using peroxidase-labeled HB Ab.

Materials and Methods

Immunofluorescent and electron microscopic studies were performed on 29 liver biopsy and 4 autopsy specimens from 16 HB Ag carriers, 2 HB Ag seropositive patients with acute and 9 with chronic hepatitis and from 6 HB Ag seronegative persons. The direct fluorescent antibody technic for HB Ag using human and animal HB Ab has been described previously.[16] It was performed on part of the specimen which had been rapidly frozen. For electron microscopy, another part of the specimen was fixed in 2.5% glutaraldehyde or in 10% formaldehyde, postfixed in 1% osmium tetroxide and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi HS7S or HS8 or RCA EMU4 electron microscope at 50 kV.

For the direct immunoelectronmicroscopy, goat HB Ab (kindly supplied by Dr. J. M. Stengle, National Institutes of Health) was absorbed with normal human serum at a ratio of 10:1. It gave, on agar gel double immunodiffusion, a single precipitin line against HB Ag-positive serum and showed complete identity with a reference HB Ab. The antibody gave identity lines with ad and ay subtypes of HB Ag but did not react with normal human serum or plasma. The antibody titer to both Hb Ag subtypes was over 1:200,000, as determined by hemagglutination assay.[18] After fractionation with ammonium sulfate, the globulin fraction was

conjugated with peroxidase (Reinheitsgrad II, Boehringer Mannheim Corporation, New York, NY) using glutaraldehyde, as described by Avrameas.[19] The peroxidase-labeled goat HB Ab was absorbed with mouse liver powder and showed a single precipitin line against HB Ag-positive serum. This line became brown after reaction with 3,3'-diaminobenzidine (DAB) containing 0.01% hydrogen peroxide.[20]

Immunoelectronmicroscopy was performed on three HB Ag-positive frozen liver biopsy specimens from 2 carriers and 1 patient with chronic hepatitis and on one HB Ag-negative liver biopsy specimen from a patient with chronic hepatitis. Cryostat-frozen sections, 6 to 10μ thick were placed on 0.5% sodium silicate-coated slides, fixed for 30 minutes in 2% paraformaldehyde, washed for 30 minutes in 0.1 M cacodylate buffer containing 7.5 g% sucrose and incubated for 60 minutes with peroxidase-labeled HB Ab, undiluted or diluted 1:10. All reactions were performed at a pH of 7.6 and at a temperature of 4 C. After 30 minutes' washing in buffer, the sections were incubated for 5 to 10 minutes in DAB containing 0.01% H_2O_2,[20] washed again, postfixed in osmium tetroxide, dehydrated and embedded in Epon 812 using inverted Beem capsules.[10] After partial polymerization for 12 to 24 hours, the Beem capsules with the Epon-embedded sections were detached by rapid cooling with dry ice.[10] After complete polymerization, the tissue in the Epon blocks was studied under the light microscope, the best areas were selected for sectioning and were examined, either unstained or after light staining with lead citrate, in the electron microscope. The following controls were employed: peroxidase-labeled rabbit anti-human IgG diluted 1:10; blocking of peroxidase-labeled Hb Ab with unlabeled Hb Ab; absorption of peroxidase-labeled HB Ab with HB Ag containing human serum at a ratio of 10:1; omission of incubation with antiserum in order to test for endogenous peroxidase activity; incubation with saline containing 10 mg peroxidase/ml.

Electron Microscopy

Circular and Tubular Structures

The hepatocytic cytoplasm of HB Ag-positive persons contained circular and tubular structures within the cisternae of the endoplasmic reticulum (ER) (Figure 1). Some cells were packed with these structures; in others they were only present in part of the cytoplasm or scattered throughout. They were only moderately electron scattering and had a poorly defined outline. No surface subunits could be disclosed on high magnification (Figure 2). The circular bodies measured 20 to 30 nm in diameter and sometimes contained a dark dot within the electron-lucent center (Figure 3). The tubules had a similar diameter but varied in length. They were straight or curved and were oriented parallel to the long axis of the ER, although sometimes they crossed each other. In oblique sections, ellipsoid structures were seen, suggesting that the circular bodies are cross sections of the tubules (Figure 2). One to more than ten of these bodies were observed within the same cisterna and were usually accompanied by a flocculant material. No budding from or attachment to the membranes was observed. The circular and

tubular structures were not seen in the Golgi cisternae, in the nuclei or free in the cytoplasm.

Surrounding Membrane and Cell Organelles

The surrounding membrane of the ER was smooth; occasionally, however, ribosomes were attached to it (Figure 4). The smooth ER was in excess, dilated and contained amorphous flocculant material in many hepatocytes including those cisternae which did not contain the bodies. Glycogen varied in amount. The mitochondria showed variation in size and shape, occasionally contained crystals and sometimes appeared to be displaced by the increased smooth ER. The rough ER seemed to be decreased in amount. Many free ribosomes were present in the cytoplasm. Many hepatocytes contained fat droplets and lysosomes, including autophagic vacuoles. No necrotic hepatocytes containing the circular and tubular structures were seen. Microbodies varied in number. The Golgi apparatus and bile canaliculi did not appear to be altered. The nuclei often were small, with an irregular membrane, or they were absent from the plane of section of the hepatocytes containing the intracisternal bodies. Sometimes the nuclei were double. In one case with many cytoplasmic inclusions within the nuclei, the intracisternal bodies were seen within the inclusion.

Distribution and Incidence

The percentage of hepatocytes containing the circular and tubular structures varied from specimen to specimen, ranging from 5% to virtually all hepatocytes observed. These cells were in clusters or scattered throughout. Their percentage corresponded roughly to the percentage of positive cells after staining of the frozen part of the specimen with fluoresceinated HB Ab. The intracisternal bodies were present in 18 of 27 HB Ag-positive specimens. Thirteen of these 18 specimens were from carriers, four from patients with chronic hepatitis and one from a patient with acute hepatitis. In the nine specimens where they were not found, only scattered hepatocytes with HB Ag-specific cytoplasmic fluorescence were observed. Five of these specimens were obtained from patients with chronic hepatitis, three from carriers and one from a patient with acute hepatitis.

In nine specimens, seven from patients with chronic hepatitis and two from carriers, 20- to 25-nm spherical intranuclear particles, which have been well documented in the literature,[5] were seen. In four of these specimens, three from patients with chronic hepatitis and one from a carrier, both the intranuclear particles and the intracisternal bodies were

seen, sometimes within the same cell (Figure 5). Neither intranuclear particles nor intracisternal bodies could be found in the six HB Ag-negative specimens and in four HB Ag-positive specimens from 2 carriers, 1 patient with acute and 1 with chronic hepatitis.

Immunoelectronmicroscopy

After incubation with peroxidase-labeled HB Ab, the electron-dense reaction product localized along the circular and tubular structures and the surrounding membrane of ER (Figures 6–8). The deposits in both locations were irregular and discontinuous, but appeared to be denser along the membranes than along the enclosed bodies. They were particularly prominent in the specimens from the 2 carriers. Although freezing artifacts were present, the other cell organelles could be easily recognized and were free of reaction product except for scattered cytoplasmic vacuoles. The latter had endogenous peroxidase activity and were probably distorted peroxisomes. The rough ER showed a weak reaction with peroxidase-labeled HB Ab. This reaction was considered to be nonspecific since a similar reaction was observed with peroxidase-labeled antihuman IgG and with peroxidase-containing saline. The reaction of the smooth ER and the enclosed bodies with peroxidase-labeled HB Ab was abolished after absorption of the antibody with HB Ag containing serum (Figure 9) and after blocking with unlabeled HB Ab (Figure 10). These sites did not react with peroxidase-labeled antihuman IgG and did not have endogenous (Figure 11) or exogenous peroxidase activity. The HB Ag-negative liver biopsy specimen did not contain any circular and tubular structures. After incubation with peroxidase-labeled HB Ab, reaction product was seen only around cytoplasmic vacuoles, similar to those described above.

Discussion

The findings reported herein indicate that the tubular and circular bodies with the surrounding membrane of ER represent an ultrastructural form of HB Ag in the hepatocytic cytoplasm. On light microscopy, the hepatocytic cytoplasm containing these bodies has a "ground-glass" appearance [21] and stains with Gomori's aldehyde fuchsin stain.[22] These hepatocytes show intense cytoplasmic fluorescence after incubation with fluoresceinated HB Ab.[21] Direct and indirect [10] immunoelectronmicroscopy using peroxidase- or ferritin-labeled antibodies indicates that the circular and tubular structures and the surrounding membranes of ER contain HB antigenic determinants.

The tubular aggregates in the ER of endothelial cells,[23] seen in human

renal diseases (particularly systemic lupus erythematosus), some neoplasms and viral infections, are clearly different in appearance. Structures similar but not identical to those shown here have been described in the ER of cotton zygotes,[24] toad hypophyseal cells [25] and liver cells of starved frogs [26] and 1 dog.[27] Stein *et al* [28] were the first to observe tubular and round particles, although slightly larger than described here, in the cytoplasm of hepatocytes of patients or carriers with HB antigenemia. Their findings were confirmed by us [29] and others.[30,31,8] Direct proof that these bodies contain HB antigenic determinants is provided here by binding of peroxidase-labeled HB Ab, confirming our previous results by indirect immunoelectronmicroscopy. A comparison with the virus-like particles in the feces of patients with hepatitis A is not possible because of the differences in preparation of the specimens.[32]

The finding of these structures that are antigenically related to HB Ag raises several questions. What is the site of formation of these bodies? What is the relation to the infectious agent of hepatitis B? As to the site of formation, the smooth ER seems to play the major role because: the bodies are found exclusively in this location; the amount of smooth ER is increased in the hepatocytes containing the bodies and the agranular membranes themselves react with HB Ab. Since there is no evidence that the smooth ER synthesizes proteins, we have to assume that the HB Ag-containing material is manufactured elsewhere in the hepatocytes, possibly in the rough ER, and that it accumulates in the smooth ER, resulting in visible tubular and circular bodies. Perhaps the microsomal enzyme system participates in this process. The Golgi apparatus does not seem to be involved. Morphologically the cells containing the bodies are characteristic and resemble the "induction cells," described by Klinge *et al*,[33] in persons who had received drugs which are handled by the microsomal biotransformation system.

As to the relation to the infectious agent of hepatitis B, Stein *et al* [28] suggested that these structures represent the intrahepatic form of the hepatitis B virus (HBV). However, the variable morphology of these bodies argues against this suggestion. In addition, they resemble the tubules found in the serum of patients with HB antigenemia which presumably represent excess coat material.[34] Furthermore, the antigenicity of the cytoplasmic structures seems to be different from that of the nuclear particles,[14] which probably represent the internal core of the mature HBV. Therefore, we favor the assumption that the tubular and circular structures are virally coded coat material of HBV produced in infected hepatocytes. Immunoelectronmicroscopy using antibodies spe-

cific for the core and the coat of HBV should demonstrate whether this assumption is correct.

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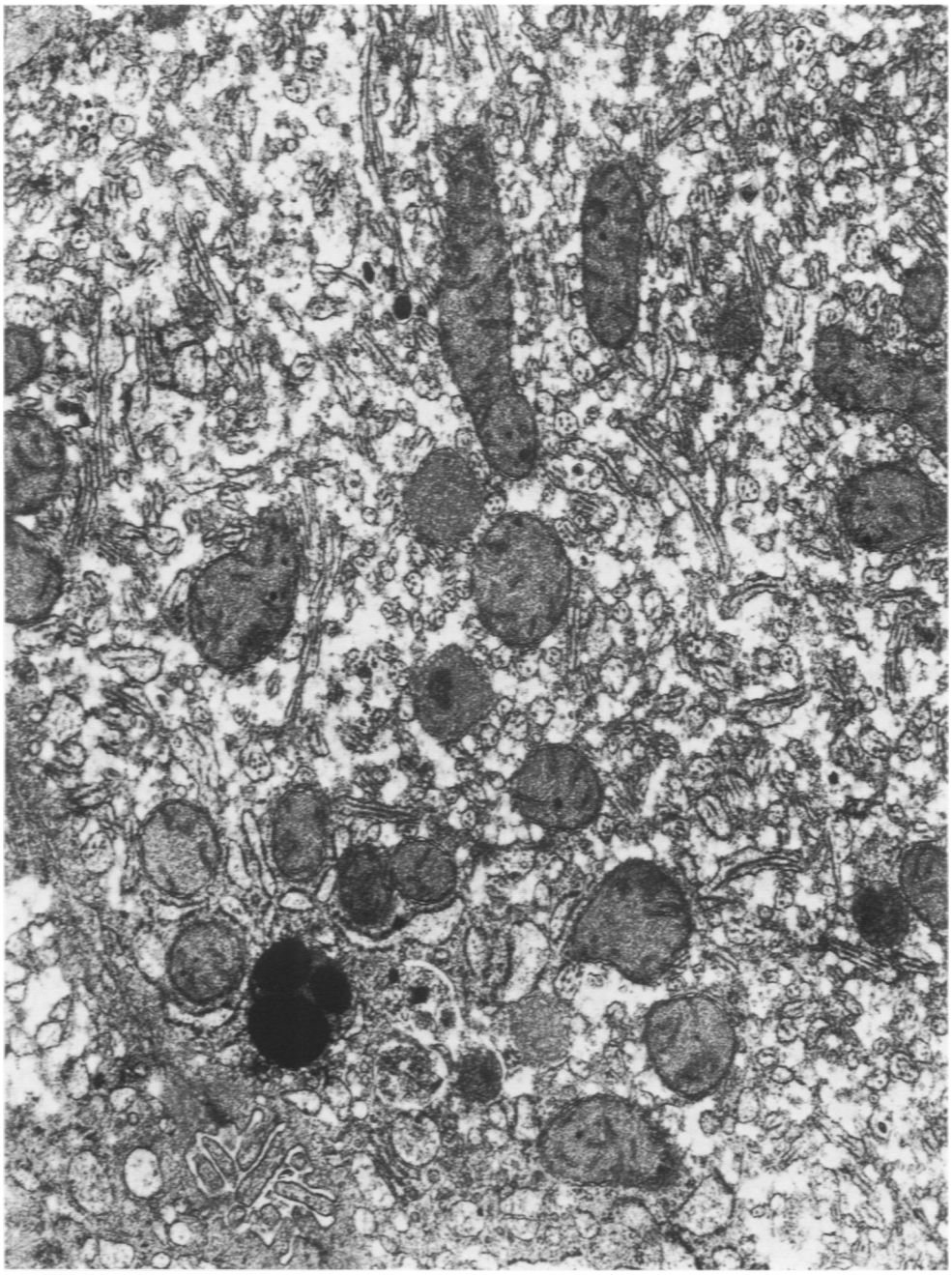


Fig 1—The hepatocyte of an HB Ag carrier contains numerous tubular and circular structures within the cisternae of the endoplasmic reticulum (Uranyl acetate and lead citrate, × 12,000).

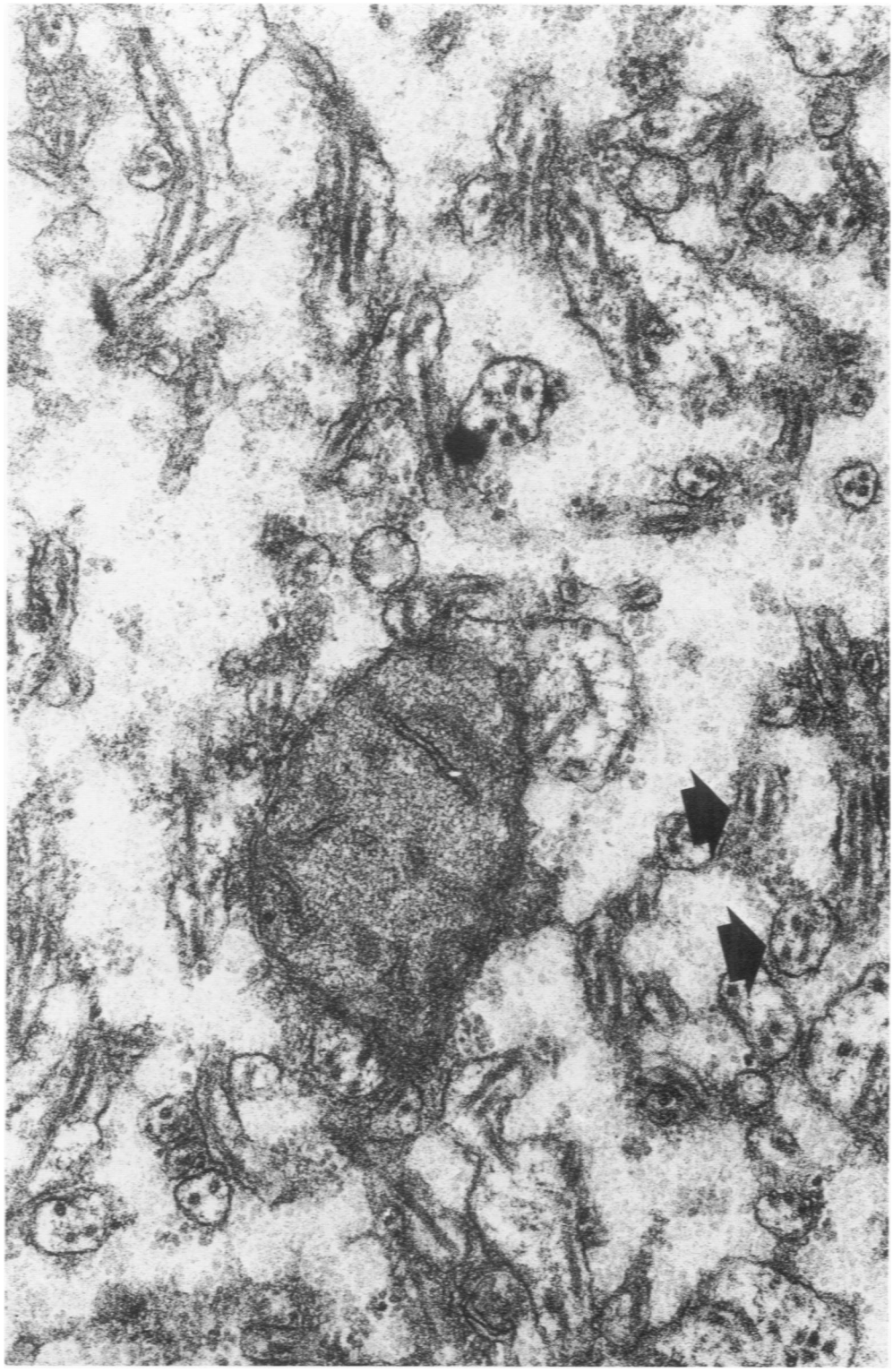
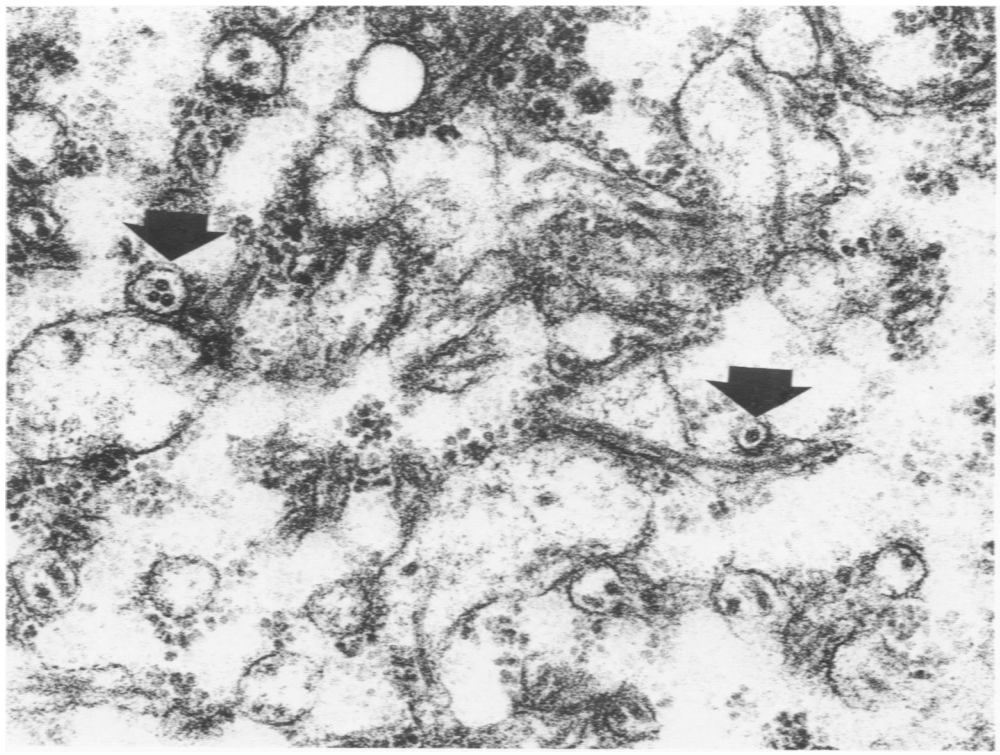


Fig 2—Higher magnification does not reveal any subunits of the intracisternal bodies; in oblique sections they appear ellipsoid (*arrows*) (Uranyl acetate and lead citrate, × 54,000).



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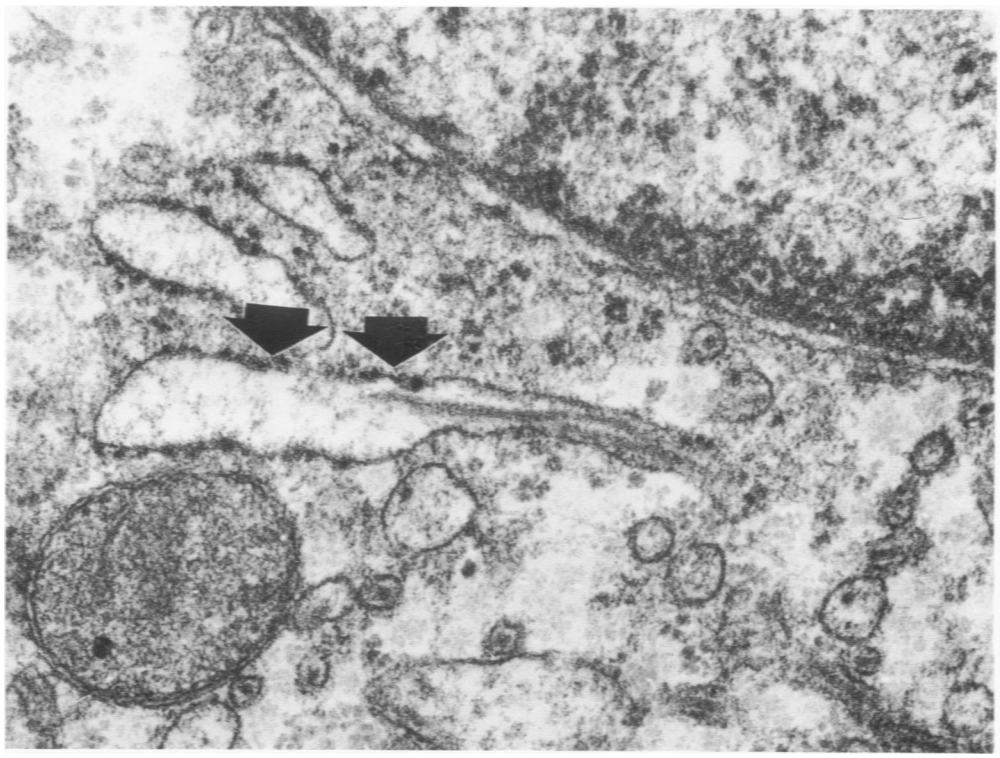

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Fig 3—Some circular structures contain a dark dot within the electron-lucent center (*arrows*) (Uranyl acetate and lead citrate, × 86,000). **Fig 4**—The membrane surrounding the intracisternal bodies is predominantly smooth, however occasionally ribosomes are attached to it (*arrows*) (Uranyl acetate and lead citrate, × 90,000).

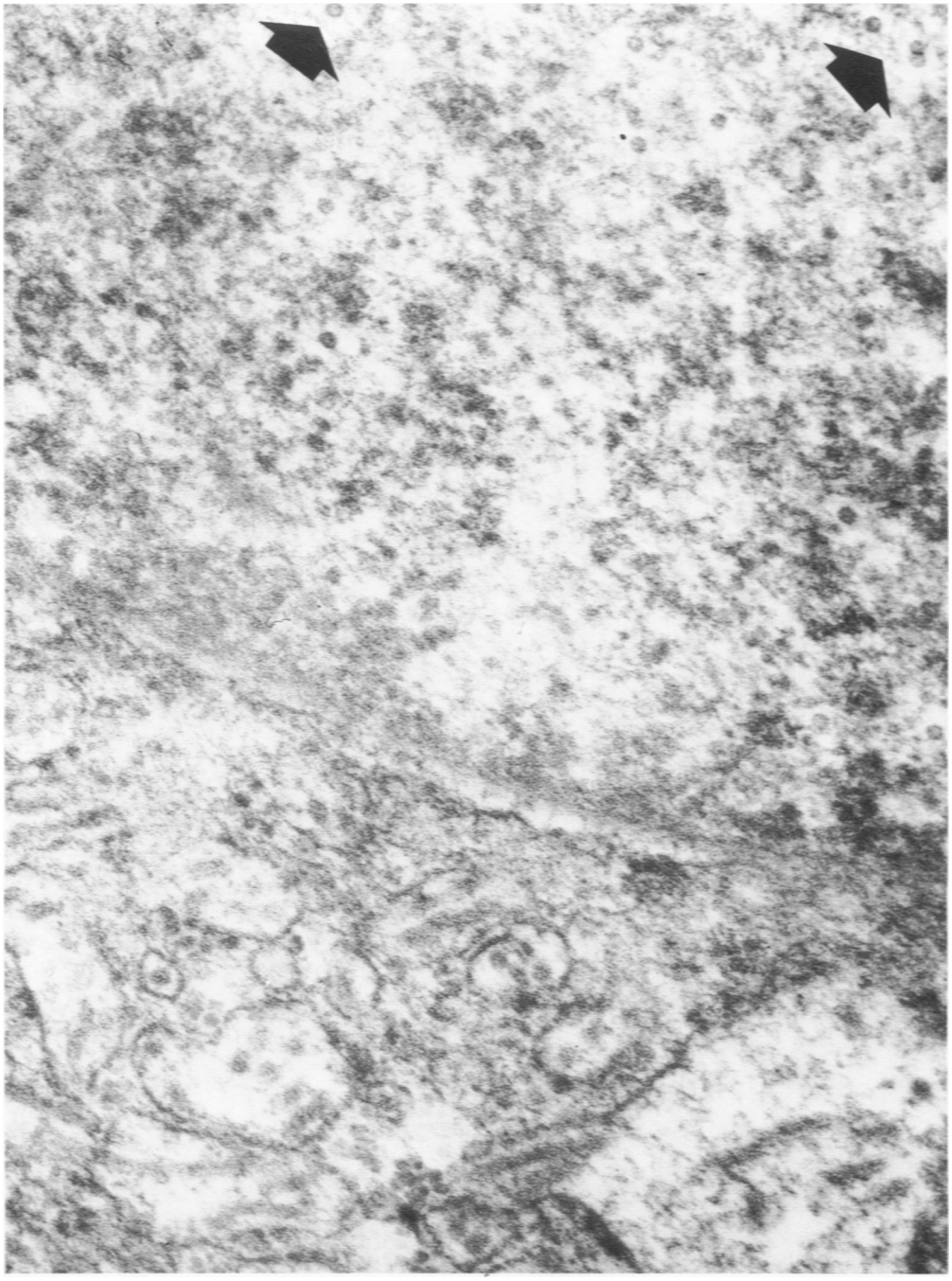
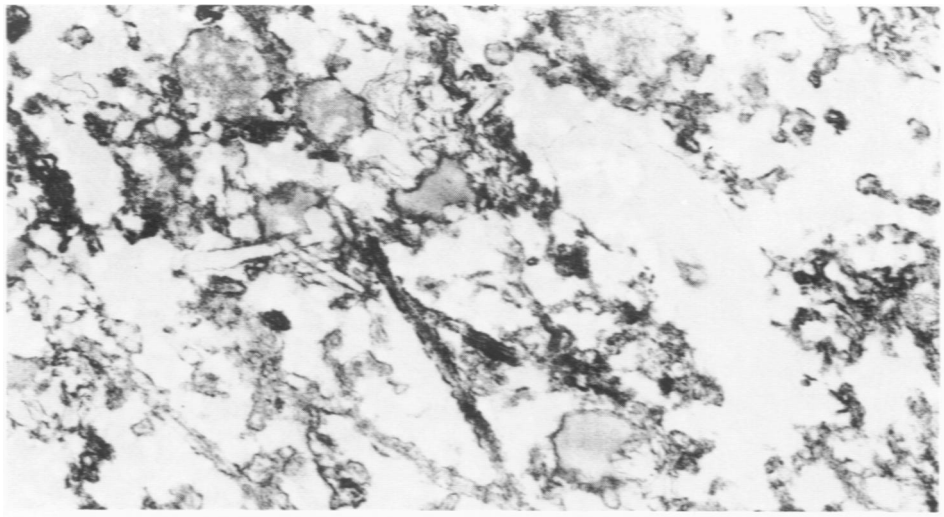


Fig 5—The hepatocyte of an HB Ag carrier contains 20-nm spherical particles in the nucleus (*arrows*) and tubular and circular bodies in the cytoplasm (Uranyl acetate and lead citrate, × 100,000).



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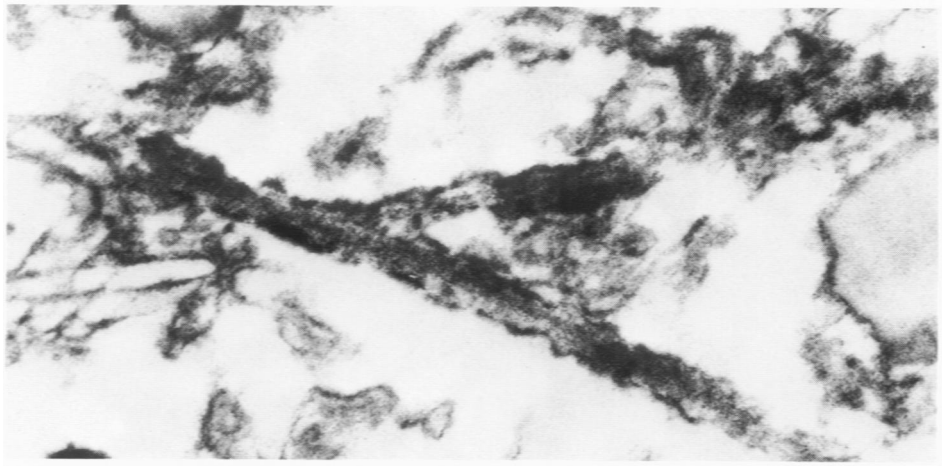

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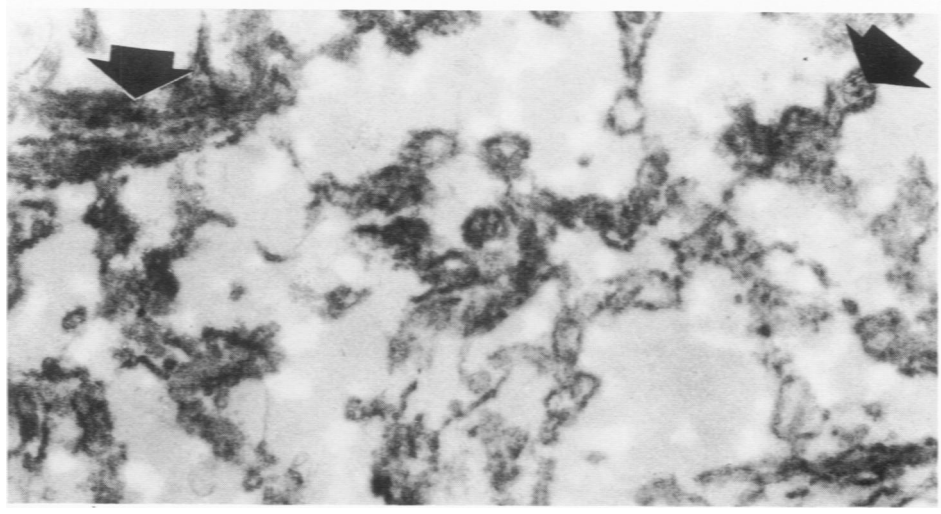

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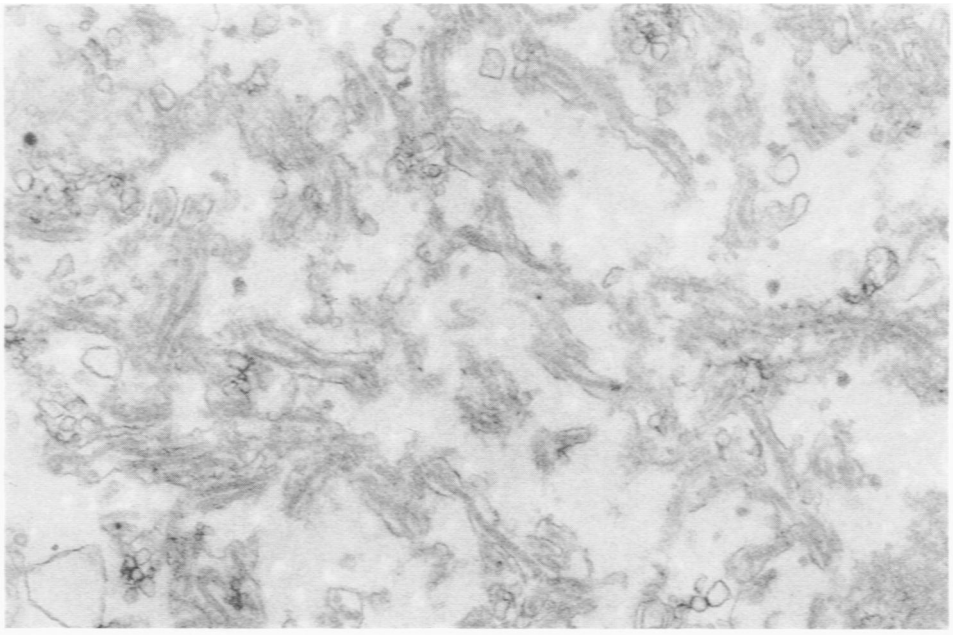
Figs 6–8—After incubation of the liver biopsy specimen from an HB Ag carrier with peroxidase-labeled HB Ab, the electron-dense reaction product localizes in the hepatocytic cytoplasm along the tubular and circular structures (*arrows*) and the surrounding membrane of endoplasmic reticulum (Light lead citrate staining, **6** × 25,000; **7** × 75,000; **8,** × 58,000).



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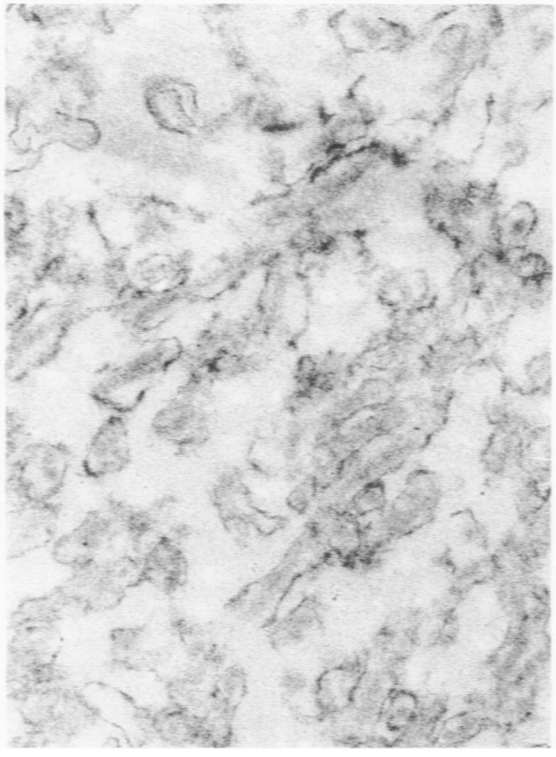

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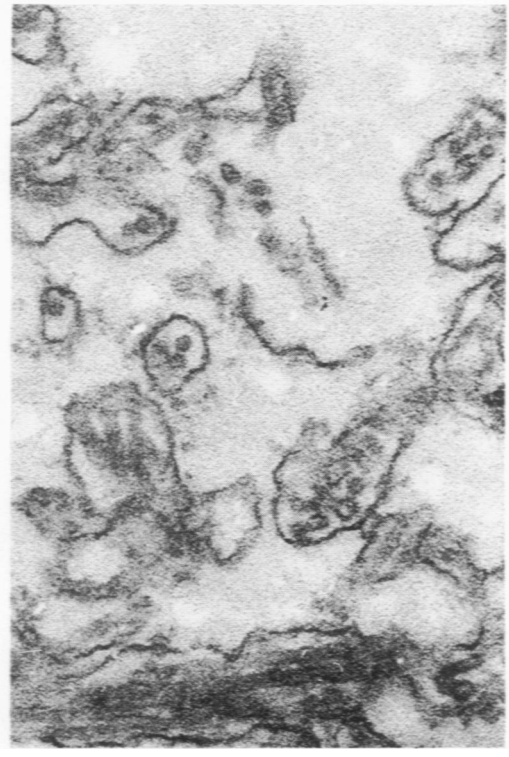

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Figs 9–11—Control reactions for the peroxidase-labeled antibody technic on the liver biopsy specimen from an HB Ag carrier. **9**—Incubation with peroxidase-labeled HB Ab which had been absorbed with Hb Ag. **10**—Blocking of peroxidase-labeled HB Ab with unlabeled HB Ab. **11**—Incubation with saline to test for endogenous peroxidase activity. No reaction product is seen after any of these control reactions (Light lead citrate staining, **9**, × 52,000; **10**, × 50,000; **11**, × 90,000).