Immuno-electron Microscopy of Hepatitis B Antigen in Liver[1] (36669)

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(Introduced by Hans Popper)

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Several investigators (1–5) found by electron microscopy 20 nm virus-like particles in the nuclei and less commonly in the cytoplasm of hepatocytes of patients with hepatitis B antigen (HB Ag) (6) (Australia antigen, hepatitis-associated antigen) in their serum. The majority of these patients had a decreased immune response (1, 3, 5) without hepatitis (1), with mild chronic persistent hepatitis (1), with chronic active hepatitis (3), or with unresolved hepatitis (5) or they were carriers with mild hepatitis (4). The liver of only one patient, a heroin addict, "revealed changes consistent with acute viral hepatitis" (2). The 20 nm particles presumably carry one or several antigenic determinants of HB Ag since similar particles found in the serum of HB Ag-positive patients (7) are aggregated by antiserum to HB Ag (HB Ab) (8). This assumption was strengthened by the binding of ferritin-labeled HB Ab to virus-like particles in the livers of two immunosuppressed patients with chronic active hepatitis (9, 10). To obtain still more support for this assumption we applied the indirect ferritin-labeled antibody technique to livers of two patients with 20 nm particles in the hepatocytic nuclei.

Material and Methods. Liver tissue from two patients with persistent HB Ag in the serum was examined. Patient LP was a 29-year-old woman with lymphatic leukemia treated with immunosuppressive drugs. Three months prior to death she developed acute viral hepatitis. She recovered although HB Ag persisted in her serum. She developed periarteritis nodosa and died from gangrene of the small bowel. At autopsy the liver showed steatosis and passive congestion, but no hepatitis. Details of the clinical and immunologic findings have been presented before (11).

Patient AE was a 74-year-old man who had received blood transfusions during resection of an abdominal aortic aneurysm infected with *Salmonella choleraesuis*. Ten months later he developed HB Ag-positive hepatitis. Since then, HB Ag and also Salmonella persisted in his blood. The patient developed chronic hepatitis with several episodes of hepatic encephalopathy and died in hepatic coma 17 months after the operation. At necropsy he had chronic aggressive hepatitis and cirrhosis.

Liver tissue obtained at autopsy was frozen in dry ice and isopentane and stored at −80°. For demonstration of HB Ag by the direct fluorescent antibody technique, 4 μ thick cryostat sections were air-dried and used unfixed or were fixed in acetone or 2% paraformaldehyde for 10 min and incubated with fluoresceinated HB Ab for 30 min. The specificity of HB Ab obtained from two patients with hemophilia, was established by immunodiffusion, by absorption of HB Ab by HB Ag-containing serum, by blocking of fluoresceinated HB Ab by nonfluoresceinated HB Ab and by examination of normal human liver with fluoresceinated HB Ab.

For electron microscopy, formaldehyde-fixed tissue was postfixed in 1% osmium tetroxide, dehydrated and embedded in Epon 812. Ultrathin sections were cut with an

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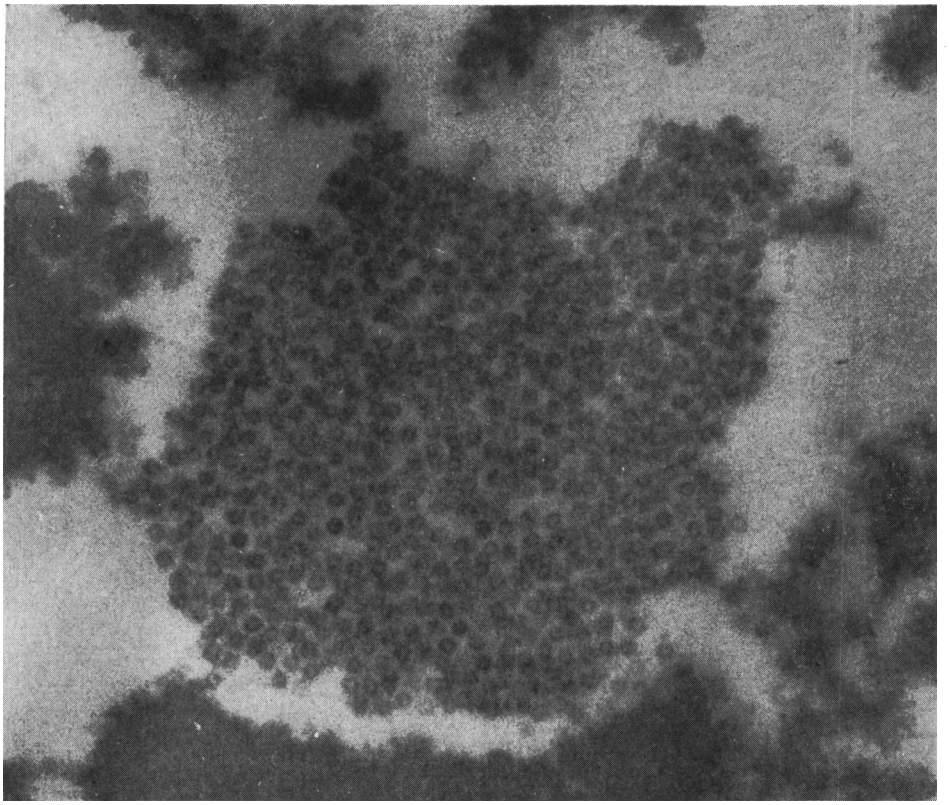

FIG. 1. Large aggregate of virus-like particles in a hepatocytic nucleus of patient LP (×115,000, lead citrate).

LKB Ultrotome, stained with lead citrate and examined with a Hitachi HS7S electron microscope.

For immuno-electron microscopy, 16 μ thick frozen sections measuring approximately 0.3 by 0.6 cm were placed on 0.5% sodium silicate-coated slides and fixed in 2% paraformaldehyde for 2 to 5 min. The sections were washed in 0.1 *M* cacodylate buffer, incubated with HB Ab for 20 min, washed, incubated with ferritin-labeled goat anti-human IgG undiluted or diluted 1:10 (Cappel Laboratories, Inc., Downington, PA), washed again and postfixed in 1% osmium tetroxide. The sections, still attached to the glass slides, were dehydrated and embedded in Epon 812 with inverted Beem (Ladd Research, Burlington, VT) capsules glued around the sections to the slides with Duco Cement (E. I. duPont deNemours and Co., Wilmington, DE). After 24 hr polymerization, the Beem capsules with the Epon-embedded sections could be easily detached from the slides by rapid cooling with dry ice. After completion of the polymerization the specimens were cut and stained for electron microscopy. As control, a normal human liver was processed for electron microscopy and immuno-electron microscopy in the same way and a specimen of patient AE was treated with normal human serum instead of HB Ab.

Results. Immunofluorescence microscopy. The nuclei of almost all hepatocytes in the liver tissue of patient LP contained discrete fluorescent granules after staining with fluoresceinated HB Ab indicating the presence of HB Ag. The cytoplasm of occasional liver cells showed diffuse fluorescence. The majority of hepatocytic nuclei in the cirrhotic nodules of the liver of patient AE exhibited diffuse staining with fluoresceinated HB Ab. Interstitial cells in the nodules and

septa, presumably Kupffer cells and inflammatory cells, were also stained. Similar results were obtained with fixed and unfixed tissue. The normal human liver showed no specific fluorescence. Absorption or blocking of fluoresceinated HB Ab abolished the fluorescence of HB Ag-containing structures.

Electron microscopy. More than 50% of the hepatocytic nuclei in the specimen of patient LP contained numerous uniform round virus-like particles, measuring 18–25 nm in diameter (Fig. 1). They were usually empty, but some particles had an electron dense central core. Their membrane seemed to be composed of subunits. These particles occurred both in large groups and scattered in the nucleoplasm between the nuclear chromatin. By contrast, the particles in the hepatocytic nuclei of patient AE were randomly dispersed in the nucleoplasm and formed occasional chains, but no groups (Fig. 2). The particles were also present in more than half of the hepatocytic nuclei of this patient and were identical in shape and size to those seen in patient LP. The integrity of the hepatocytes could not be evaluated because of postmortal changes. In both patients, no particles were seen in the cytoplasm of hepatocytes or in mesenchymal cells. Tubular forms and 42 nm particles (Dane) were absent from both cases. The normal human liver did not contain any virus-like particles.

Immuno-electron microscopy. Similar results were obtained after incubation of liver tissue of patients LP and AE with HB Ab followed by ferritin-labeled anti-human IgG. Aggregates of virus-like particles in hepatocytic nuclei were surrounded by a rim of ferritin granules identified as the typical tetrads on high magnification (Figs. 3 and 4). Ferritin granules were also within the aggregates in close proximity to the particles. The latter were embedded in a moderately electron-dense material and were therefore much less distinct than in specimens without the addition of antisera. In patient AE the parti-

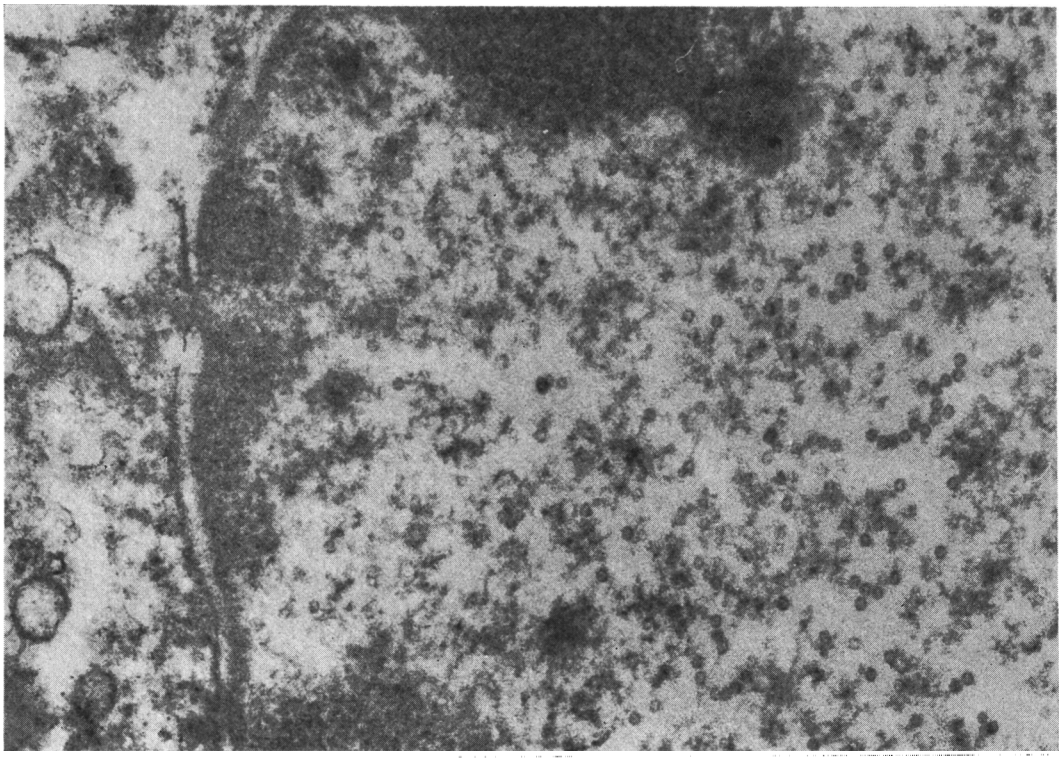

FIG. 2. The virus-like particles dispersed or in chains in a hepatocytic nucleus of patient AE (×65,000, lead citrate).

FIG. 3. An aggregate of virus-like particles in a hepatocytic nucleus of patient LP surrounded by ferritin granules after performing the indirect ferritin-labeled antibody technique with antiserum to hepatitis B antigen (×135,000, lead citrate).

cles which appeared dispersed before the addition of HB Ab were aggregated into clumps, but the number of particles in the aggregates was smaller than in patient LP. No aggregates of virus-like particles with binding of ferritin granules were seen in the cytoplasm of hepatocytes or in mesenchymal cells of patients LP and AE or in the normal human liver. The two different antisera to HB Ag gave identical results. After replacement of HB Ab by normal human serum during the staining procedure the virus-like particles in the hepatocytic nuclei of patient AE were not aggregated and ferritin granules were not bound to the particles.

Discussion. HB Ab bound specifically to virus-like particles and aggregated them in the hepatocytic nuclei of two patients with HB Ag in their serum as demonstrated by the indirect ferritin-labeled antibody technique. These findings provide strong evidence that the 20 nm particles carry at least one antigenic determinant of HB Ag. Huang *et al.* (9, 10) had similar results with the direct ferritin-labeled antibody technique. These investigators studied liver biopsies from two renal transplant patients who developed chronic aggressive hepatitis associated with persistence of HB Ag while under immunosuppressive therapy. They noted agglutination of the intranuclear and cytoplasmic virus-like particles in fresh-minced or frozen-thawed liver biopsies after treatment with ferritin-conjugated HB Ab and explained this phenomenon as migration and precipitation of the particles in the gel-like nucleoplasm akin to agar gel immunodiffusion. Apparently this migration is still possible after short fixation as seen in our preparations. Whether the HB Ag carrying particles are the hepatitis virus and the role they play in the pathogenesis of hepatitis are not known. The demonstration of the particles in both an HB Ag carrier and a patient with chronic aggressive hepatitis and cirrhosis, suggests that they do not necessarily cause hepatitis. This

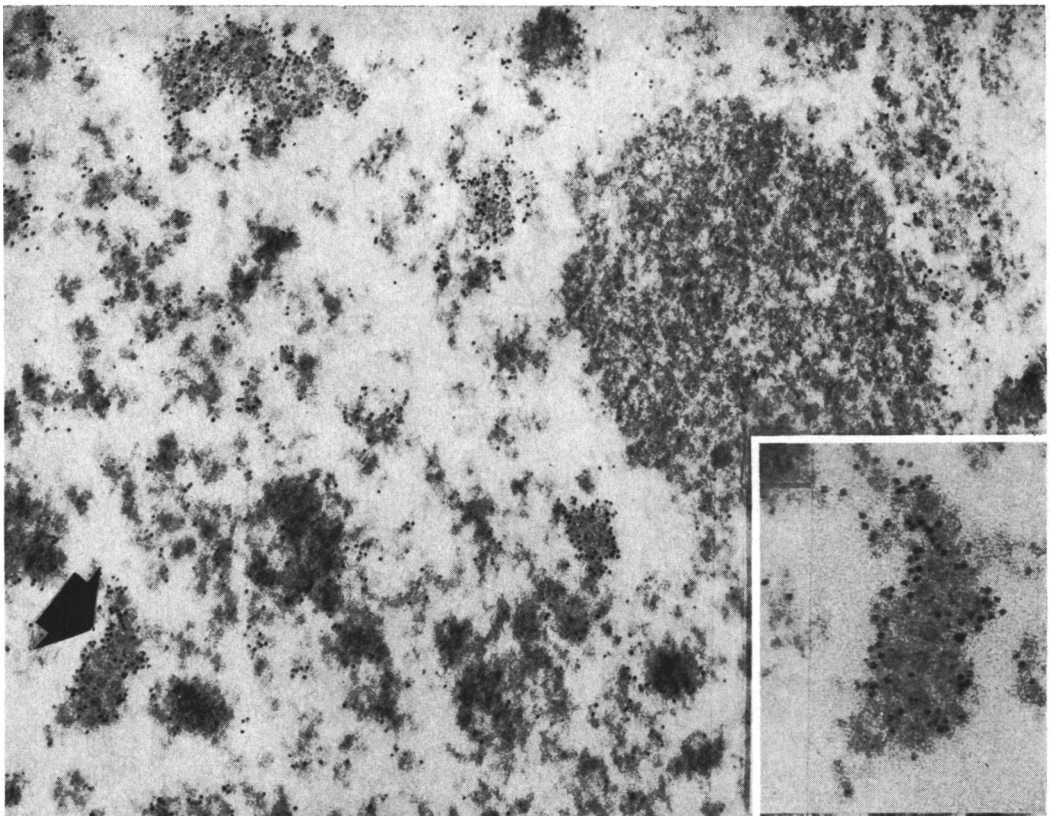

FIG. 4. The virus-like particles in a hepatocytic nucleus of patient AE aggregated and surrounded by ferritin granules after application of antiserum to hepatitis B antigen followed by ferritin-labeled anti-IgG (×55,000, lead citrate). The aggregate marked by an arrow is shown in detail in the insert (×112,000, lead citrate).

is supported by the fact that only few cases with virus-like particles in liver tissue of patients with acute viral hepatitis have been reported (*2*, 12–14). Liver injury in acute and chronic hepatitis may be mediated by cellular immunity to HB Ag or by one of the antigen–antibody systems, *i.e.*, antigen of the inner component of the 42 nm particle (Dane) or of its outer coat with specific antibody (15).

The 20 nm particles in the hepatocytic nuclei may correspond to the 20 nm particles in the serum of HB Ag positive patients or to the inner component of the 42 nm particle (Dane) as suggested by Almeida (16). The method described here might help in the demonstration of sparse HB Ag-carrying particles or HB Ag not associated with particles possibly present in acute viral hepatitis and in the investigation of the antigenic relationship of the various morphologic forms of HB Ag in liver tissue and serum.

Summary. Liver tissue from a hepatitis B antigen (HB Ag) carrier and a patient with HB Ag-positive chronic aggressive hepatitis and cirrhosis was examined for HB Ag. The antigen was detected in the nuclei of many hepatocytes by direct fluorescence antibody technique. Uniform 20 nm particles were seen in hepatocytic nuclei under the electron microscope. The particles were aggregated and surrounded by ferritin granules using the indirect ferritin-labeled antibody technique with specific antiserum to HB Ag. These findings indicate that the 20 nm particles carry at least one antigenic determinant of HB Ag.

1. Nowoslawski, A., Brzosko, W. J., Madalinski, K., and Krawczynski, K., Lancet 1, 494 (1970).

2. Nelson, J. M., Barker, L. F., and Danovitch, S. H., Lancet 2, 773 (1970).

3. Huang, S., Amer. J. Pathol. 64, 483 (1971).

4. Ricci, G., DeBac, C., and Caramia, F., Lancet 1, 494 (1972).

5. Dunn, A. E. G., Peters, R. L., Schweitzer, I. L., and Spears, R. L., Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31, 636 (1972) Abstr.).

6. Committee on Hepatitis, U.S. National Academy of Sciences, National Research Council, in press.

7. Bayer, M. E., Blumberg, B. S., and Werner, B., Nature (London) 218, 1057 (1968).

8. Almeida, J. D., Zuckerman, A. J., Taylor, P. E., and Waterson, A. P., Microbios. 2, 117 (1969).

9. Huang, S. N., Millman, I., O'Connell, A. P., Blumberg, B. S., Aronoff, A., and Gault, H., Gastroenterology 62, 181 (1972) (Abstr.).

10. Huang, S., Millman, I., O'Connell, A., Aronoff, A., Gault, H., and Blumberg, B. S., Amer. J. Pathol. 67, 453 (1972).

11. Gerber, M. A., Brodin, A., Steinberg, D., Vernace, S., Yang, C., and Paronetto, F., N. Engl. J. Med. 286, 14 (1972).

12. Almeida, J. D., Waterson, A. P., Trowell, J. M., and Neale, G., Microbios. 6, 145 (1970).

13. Deutsch, G. F., and Spence, L., Lancet 1, 447 (1972).

14. Stein, O., Fainaru, M., and Stein, Y., Lab. Invest. 26, 262 (1972).

15. Almeida, J. D., Rubenstein, D., and Stott, E. J., Lancet 2, 1225 (1971).

16. Almeida, J. D., Postgrad. Med. J. 47, 484 (1971).

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