Ultrastructural Study of Peripheral Blood Mononuclear Cells in Chronic Type B Hepatitis

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Many studies have suggested the infection of peripheral blood mononuclear cells (PBMCs) by hepatitis B virus (HBV) and its clinical implication in affecting the clinical status and persistence of the disease. However, there is still controversy concerning the mode of existence, replicative potential and cytopathological effect of the virus in these cells. In ultrastructural studies, three morphological forms of viral particles were detected in serum and hepatocytes of hepatitis B patients. However, none of these studies involved the PBMCs. In the present work, it was aimed to directly examine isolated PBMCs by transmission electronmicroscopy (TEM) to detect the various morphological forms of the virus and to verify any morphological alterations produced in the cells by the infection. PBMCs were isolated on ficoll-hypaque from heparinized blood of five patients with chronic hepatitis B (CHB) infection and subjected to conventional electronmicroscopy and immunoelectronmicroscopy (IEM). Three healthy subjects were included as controls. Among PBMCs, many monocytes and lymphocytes showed either degenerative or apoptotic features. Dilatation of the Golgi apparatus (GA) and endoplasmic reticulum (ER) was also encountered. Some cells displayed intracytoplasmic particulate structures localized within vacuoles and appearing in various forms. One form resembled the complete virion but was larger (100 nm in diameter) and lacking the double-shell contour. Spherical HBsAg subviral forms (25-33nm in diameter) were also observed. These structures were verified as HBV particles by IEM. Our results have provided further evidence for the presence of the replicable complete virion-like particles in PBMCs. They seem to persist, replicate and induce injury to PBMCs. This may affect the clinical outcome and persistence of the infection.

INTRODUCTION

Although hepatitis type B virus (HBV) is essentially hepatotropic, many genetic studies have evidenced its presence in peripheral blood mononuclear cells (PBMCs) (1-4). However, the replication competence and form of existence of HBV is still under debate and the subject of controversy. Kock et al. (5) could not detect the covalently closed circular (ccc) DNA, an early replicative form of HBV, in PBMCs and explained the presence of HBV DNA by adsorbed virus. On the contrary, several lines of evidence have confirmed replication of this virus in PBMCs, e.g. persistence of HBV cccDNA and RNA (a replicative intermediate) in PBMCs of chronic hepatitis B (CHB) patients (6), presence of the viral genome in an integrated form (7), incidence of an IL-6-mediated viral replication in PBMCs (8) and demonstration of differences in HBV DNA sequences in PBMCs from those in the serum pointing to independent viral replication (6, 9).

Furthermore, HBV infection of PBMCs was discovered to have functional and clinical implications. Feray et al. (10) postulated that PBMCs could be a source of reinfection after liver transplantation or interferon treatment. Other studies (4, 11) suggested the involvement of PBMC infection in the persistence of HBV and chronicity of infection. Also failure of artificial immunization in newborns of HBsAg positive mothers was related to defective production of IL-2 by infected lymphocytes (12).

In spite of these extensive genomic structural analysis studies in PBMCs, the ultrastructural aspects of the HBV particle's morphogenesis and cytopathic effects at cellular levels have not been equally described. Electronmicroscopic (EM) morphological and molecular features of the virus in hepatocytes and serum were proved to be informative with respect to the phases of virus development in various intracellular compartments (5, 13-22). In the present study, PBMCs were closely visualized at the ultrastructural level to examine such features which would also help to resolve some of the

controversy encountered during genetic analysis of the virus.

MATERIAL AND METHODS

Subjects:

Five patients with chronic type B hepatitis were included and in whom the liver enzyme ALT was elevated (more than double fold the normal level). Serum HBV DNA PCR assay recorded levels more than 500,000 copies/ ml . All patients were hepatitis C virus (HCV)- negative. Three normal healthy subjects (HBV- and HCV- negative) were also included in this study as controls.

Conventional EM of PBMCs:

Heparinized blood samples were collected and PBMCs isolated on ficoll-hypaque (3:4, FH:blood volume). After centrifugation at 2000rpm for 20 min., the ring formed at the interface was collected, washed twice in phosphate buffered saline (PBS) (pH=7.2) and was subjected to a routine EM according to Bozzola and Russell (23). The pellet was immediately fixed in 2.5% glutaraldehyde fixative (Merck, Damstadt, Germany)) in PBS for 30 min.at room temperature (RT), washed thrice in PBS and postfixed in 1% osmium tetroxide solution (OsO_4) (BDH chemicals, Poole, England) in PBS for 30 min. at 4^0C. This was followed by dehydration in ascending grades of ethyl alcohol, substitution in epoxy resin/ethanol mixture, infiltration in three washes of epoxy resin (Electron Microscopy Sciences) and finally embedded in epoxy resin capsules which were polymerized at 37^0C for 12 hours and at 60^0C for 2 days. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by transmission electronmicroscope (Joel 1200 EX II).

Immunoperoxidase EM of PBMCs:

The PBMCs were separated as described above and fixed in 1.25% glutaraldehyde solution in PBS. Following blocking endogenous peroxidase and background staining, cells were labeled by the primary anti-HBsAg antibody (BioGenex, USA) overnight at 4^0C. After washing, the revelation of the reaction was made by poly horseradish peroxidase detection system (BioGenex, USA). This was followed by postfixation in 1% OsO_4 solution in PBS for 1 hour at 4^0C and then the procedure was continued as above but without the uranyl acetate staining step.

RESULTS

EM examination of PBMCs, in four out of five patients, revealed the presence of viral-like particles localized in large vesicles or cisternae in the cytoplasm. They were demonstrated in two forms: one form appeared as a group of subviral spherical ring-shaped particles, 25-33nm in diameter, with electron translucent core and an outer dark rim (fig. 1,2 &3). Another larger form,100-120nm in diameter, was also demonstrated (fig. 1, 2 & 4). They did not correspond exactly to the structures described as Dane particles as they were much bigger (Dane particles usually measure 42nm in diameter) and lack the double shell. However, they were verified as virus type B since positively-stained particles of the same size appeared in the cytoplasm of the anti-HBsAg antibody-labeled cells (fig. 8). It is worth mentioning here that the two forms of virus were retained within intracytoplasmic cisternae of ER. No fusion of ER and cellular membranes and hence no extracellular shedding of such particles could be observed. The filamentous forms were not encountered in these cells. Also no core particles were displayed in the nuclei.

Many cells, especially those where viral-like particles were detected, displayed gross structural alterations. Degenerated cells were frequently seen with dissociated cytoplasm, large intracytoplasmic vesicles mostly enclosing viral particles, ill-defined cell organelles and minimal degenerative nuclear changes (fig.4). Other cells showed apoptotic features with nuclear chromatin condensation and margination, nuclear convolutions, widened nuclear pores, condensed cytoplasm but intact organelles and membrane (fig.1). A prominent common feature in degenerated and apoptotic cells was the augmentation and dilatation of the Golgi apparatus and ER (Fig. 5, 6, 7). Cells with normal morphology were also present.

Immunoperoxidase-labeled cells of all patients showed HBsAg positivity of intracytoplasmic particles measuring 100-120nm diameter (fig. 8). On one occasion, the

membrane revealed weakly stained localized regions (fig. 9). No signals were detected in the nucleus. The specificity of the reaction was confirmed by absence of signals in the negative control slides (fig. 10).

The PBMCs of all healthy subjects appeared normal. No particles were detected in these cells neither by routine nor by immunoelectronmicroscopy.

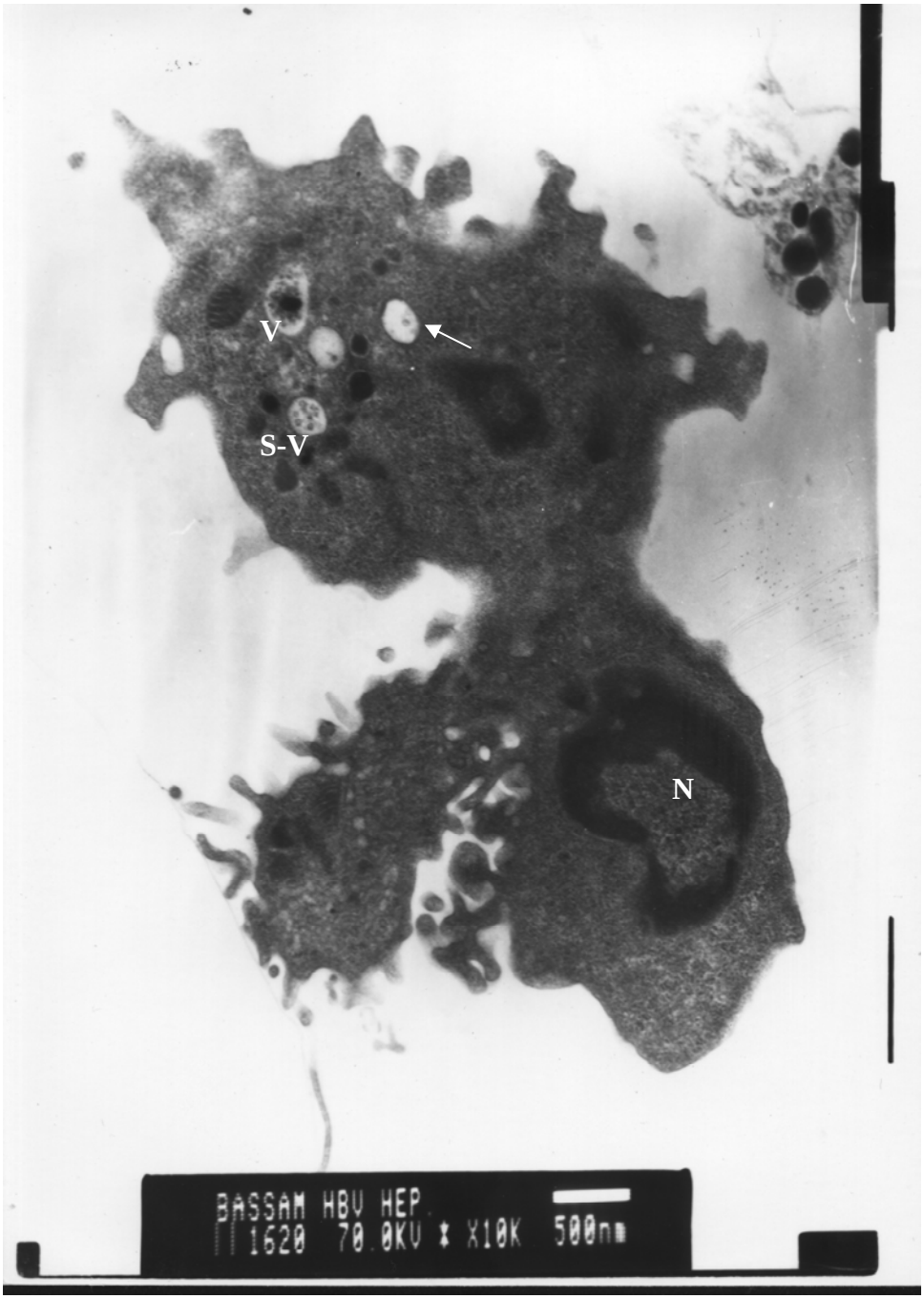

Fig. 1 A TEM photomicrograph of an apoptotic PBMC from a CHB patient. The nucleus (N) appears apoptotic with chromatin condensation and margination abutting the nuclear envelope. Intracytoplasmic dilated cisternae of ER (↑) are seen enclosing VLP of two forms: a large electron dense virion (~130nm diameter) (V) and a group of small spherical rings of subviral particles (25-30nm diameter) (S-V). The cytoplasm also displays apoptotic features entailing cytoplasmic condensation and budding with intact mitochondria and granules. X10,000

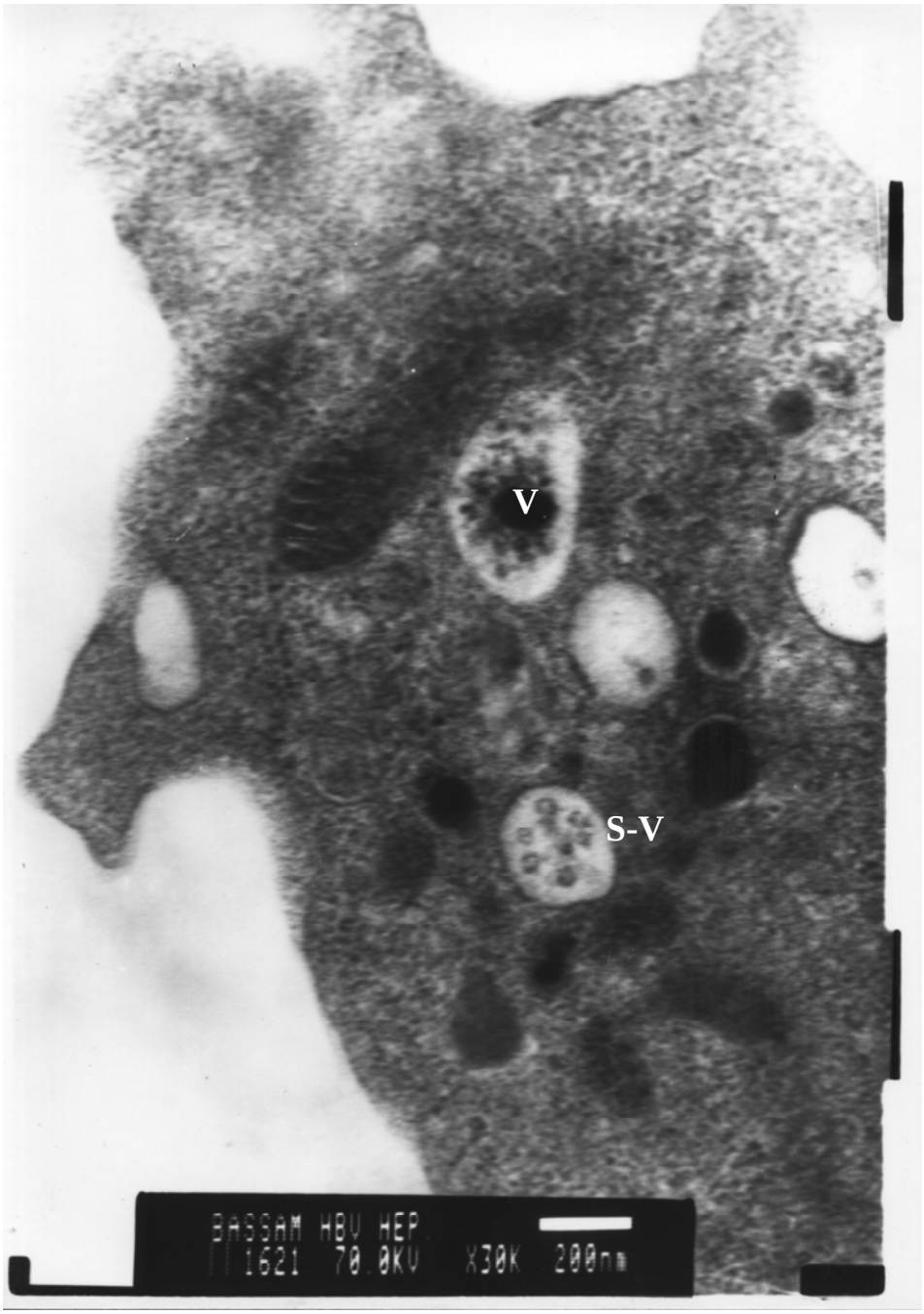

Fig. 2 A higher magnification of the previous photo revealing the two forms of intracisternal VLPs in more details: the electron dense complete virion (V) surrounded by degenerated amorphous material and a collection of smaller subviral spherical rings (S-V) with electron translucent core surrounded by a darker rim. X30,000

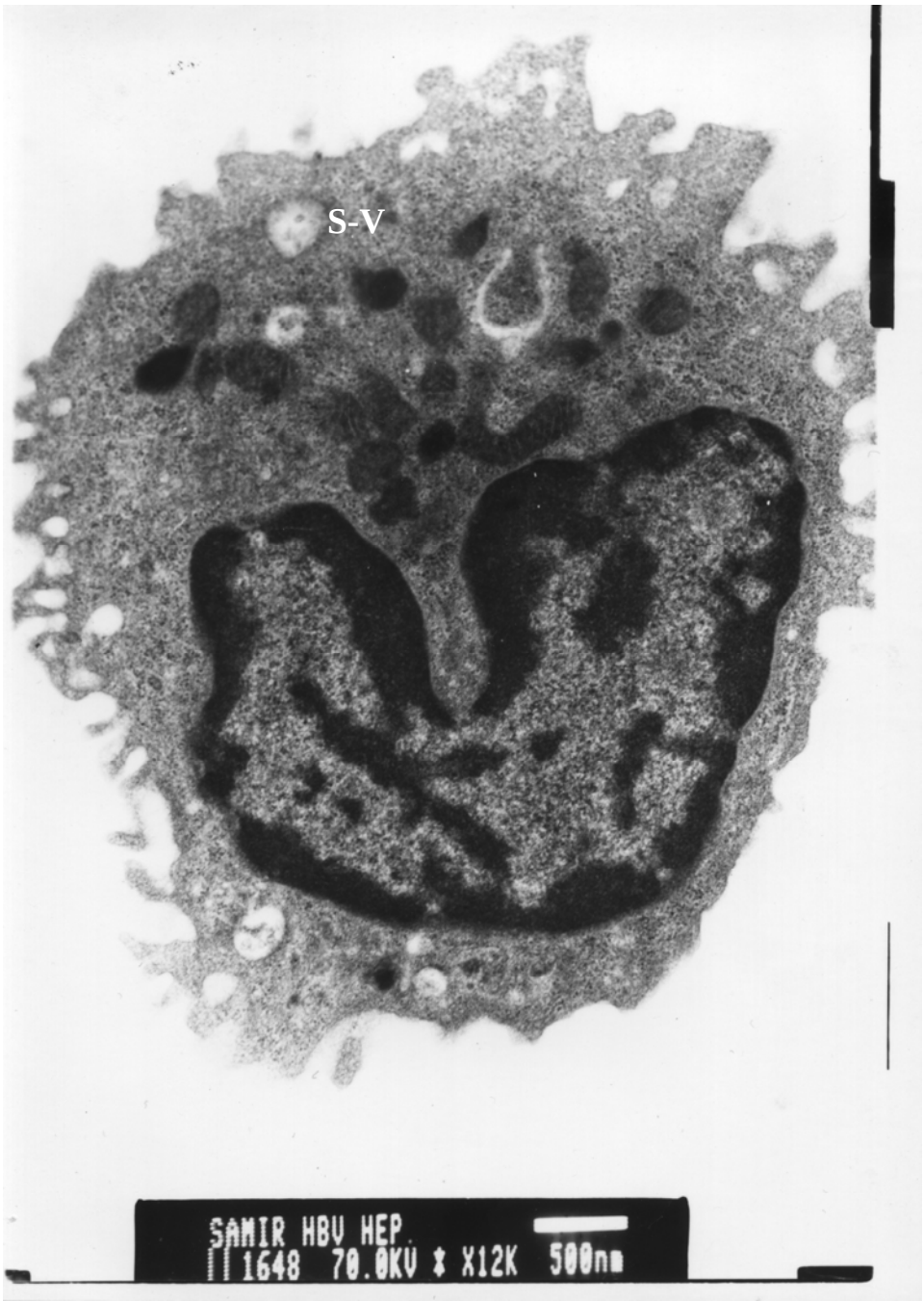

Fig. 3 A micrograph of a monocyte from another patient with CHB showing again intracisternal subviral particles (S-V). Otherwise, the cell appears morphologically normal. X12,000

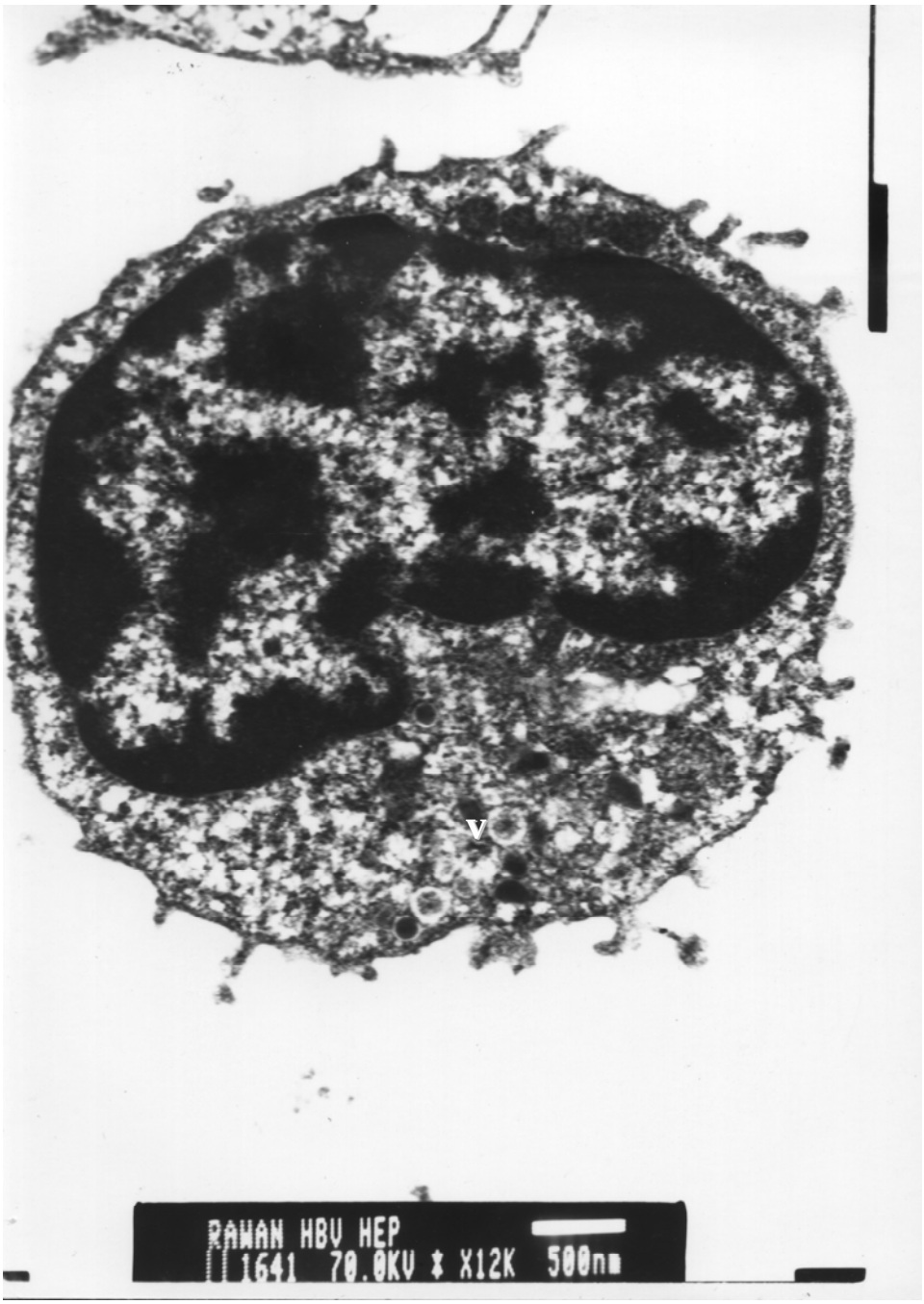

Fig. 4 In a third patient, a degenerated PBMC is shown with dissociated cytoplasm containing vesicles in which large virions (v) measuring 100nm in diameter are seen. All organelles are ill-defined. X12,000.

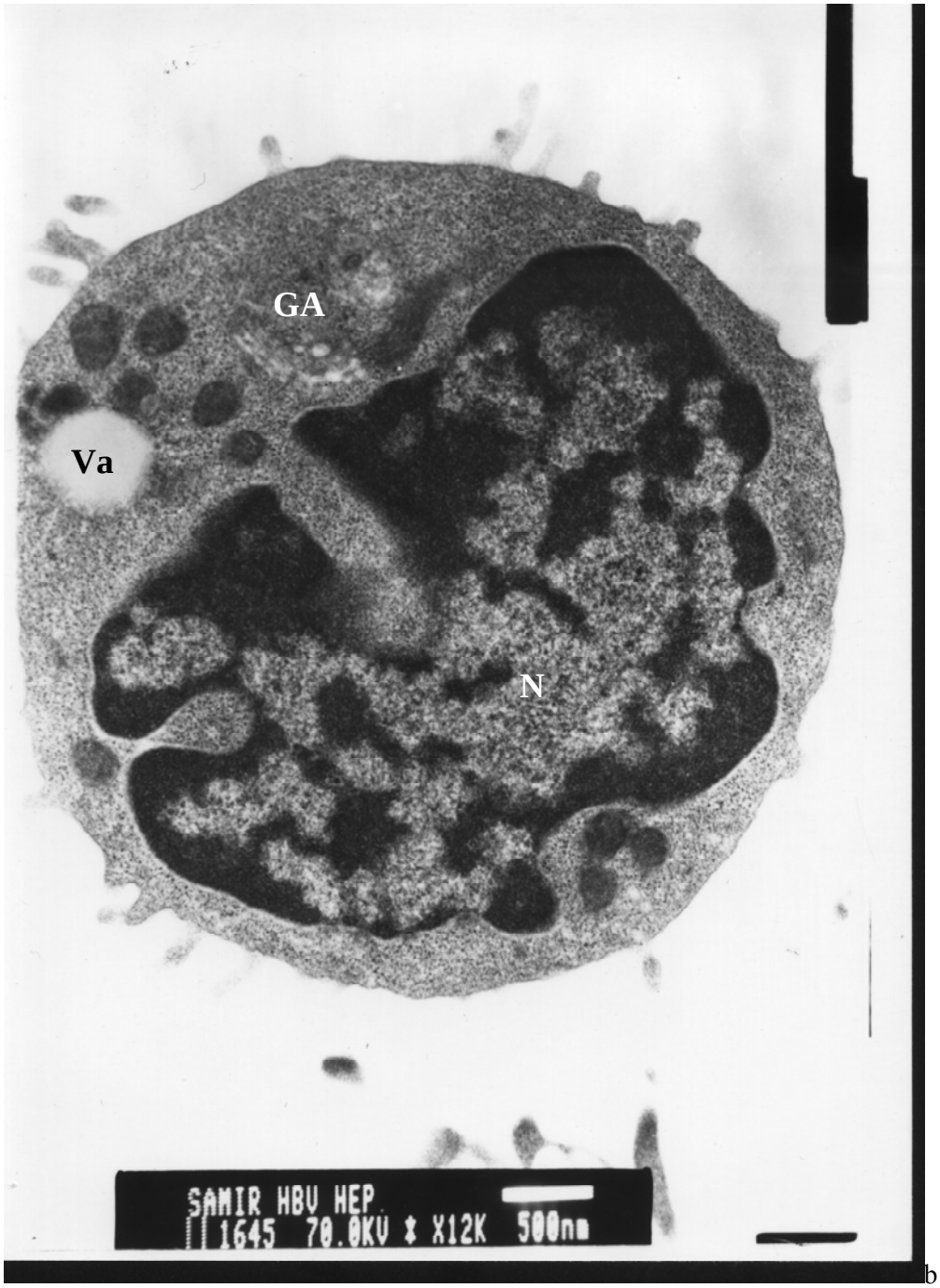

Fig. 5 A lymphocyte from an infected patient showing a big notched nucleus (N), well-defined mitochondria (M), a big empty vacuole (Va) and dilated golgi apparatus (GA). X12,000

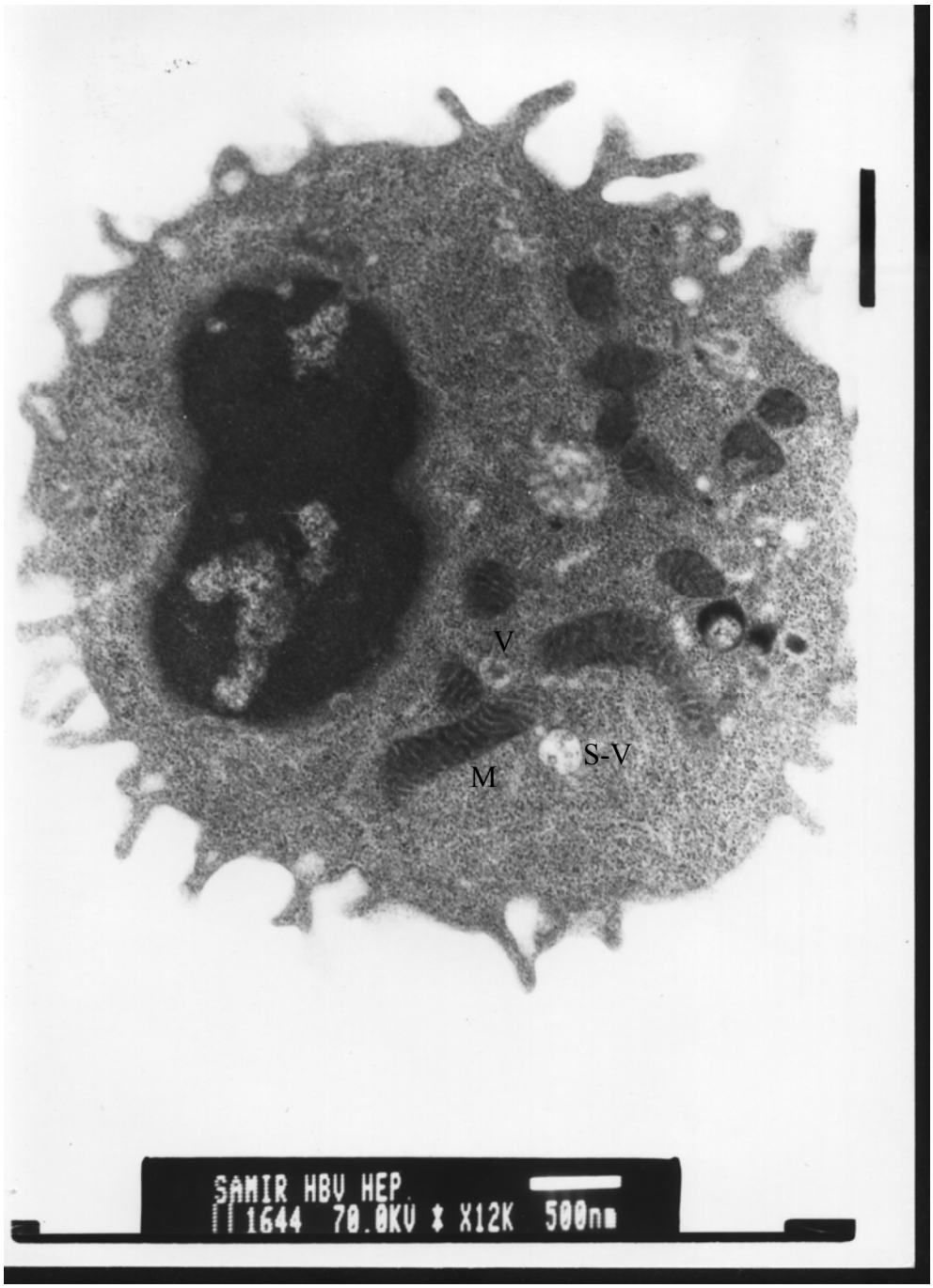

Fig. 6 An apoptotic cell from the same patient displaying a pyknotic condensed nucleus, condensed cytoplasm, intact prominent mitochondria (M), dilated GA and two intracytoplasmic vesicles containing the characteristic viral (V) and subviral (S-V) particles described in previous cells. X12,000.

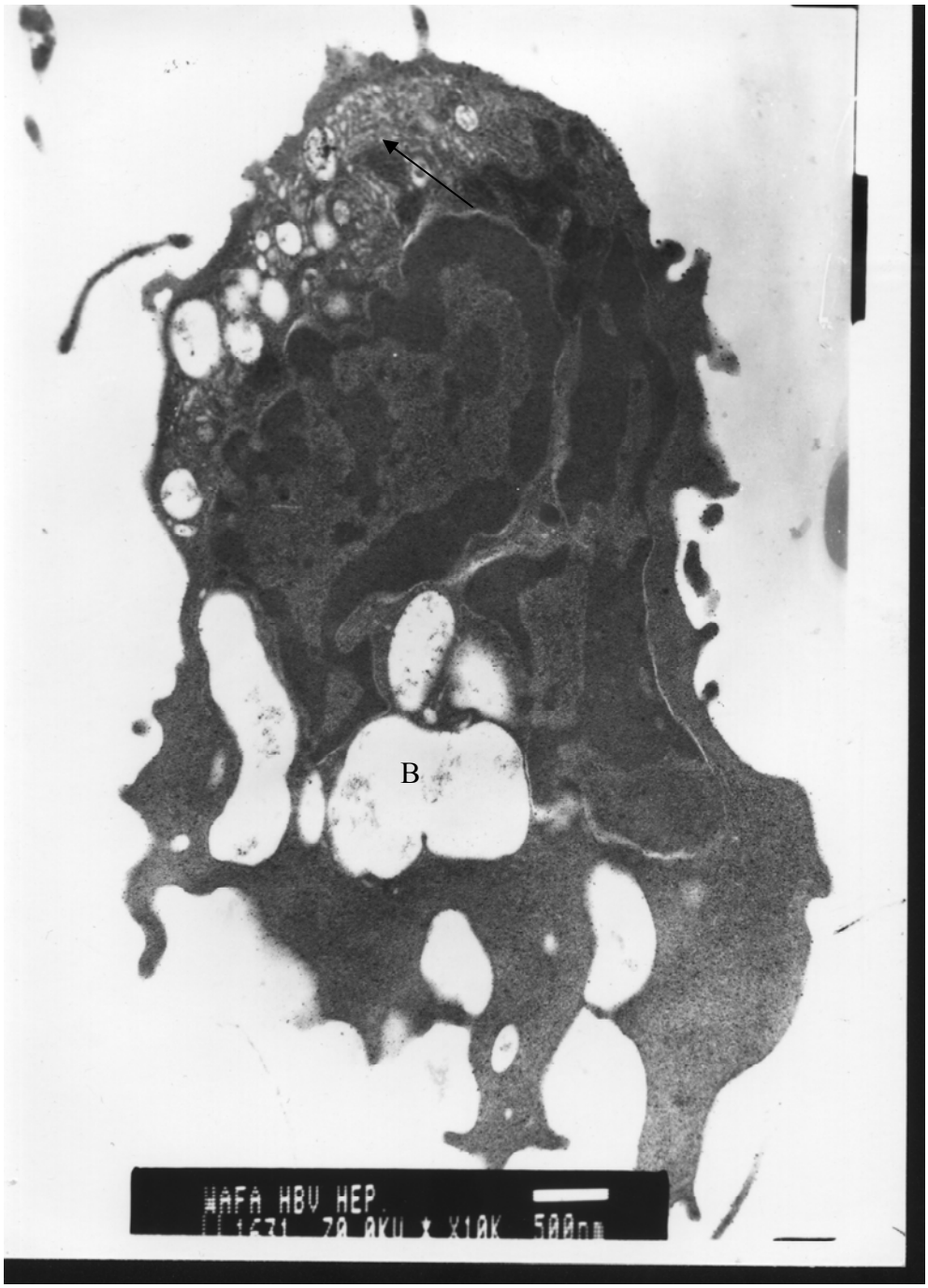

Fig 7 A photo of a PBMC from an infected patient in which the cytoplasm is condensed and studded by many bullous expansions (B) and dilatations (↑) of ER. The nucleus appears apoptotic with nuclear budding, wide nuclear pores and marginated chromatin. X10,000

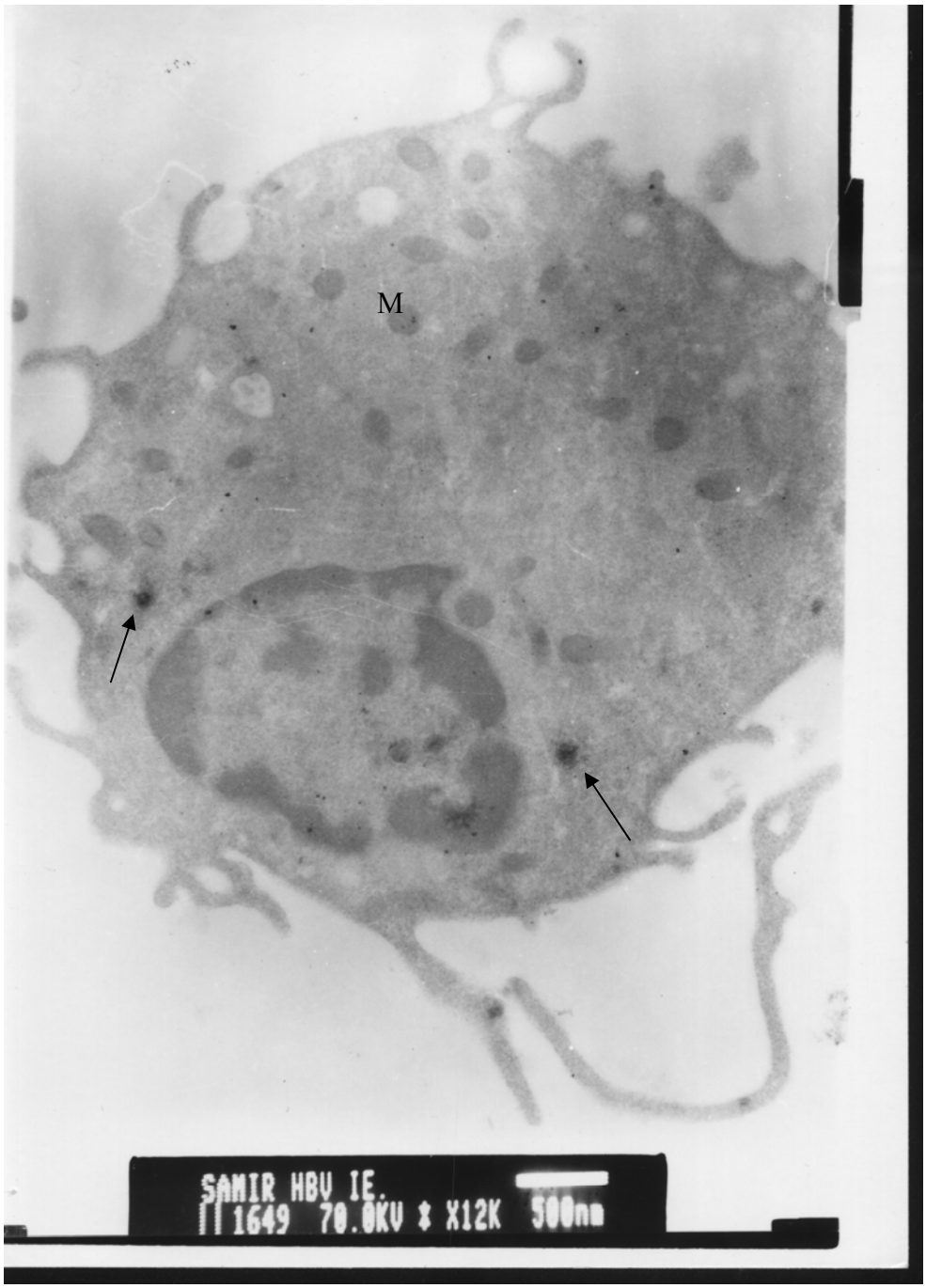

Fig. 8 A TEM photomicrograph from immunoperoxidase-labeled monocyte from a CHB patient showing HBsAg positively-stained particles (↑) (120nm in diameter) in the cytoplasm. Ghosts of mitochondria (M) are seen dispersed in the cytoplasm of the unstained cell. X12,000

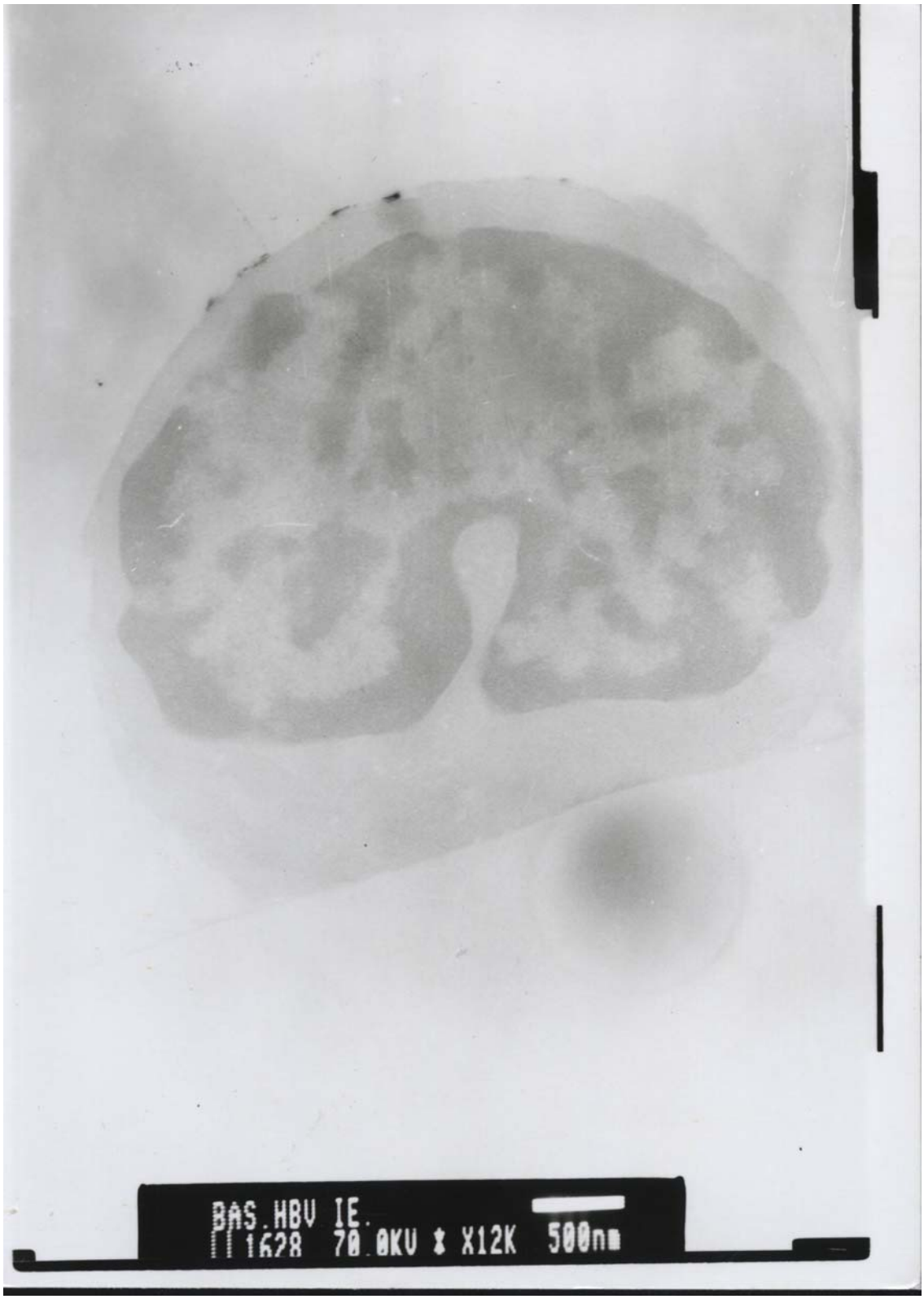

Fig. 9 Another immunoperoxidase-labeled cell from a patient revealing weak positive membraneous deposits, but the cytoplasm is free from any positively-stained bodies. X12,000

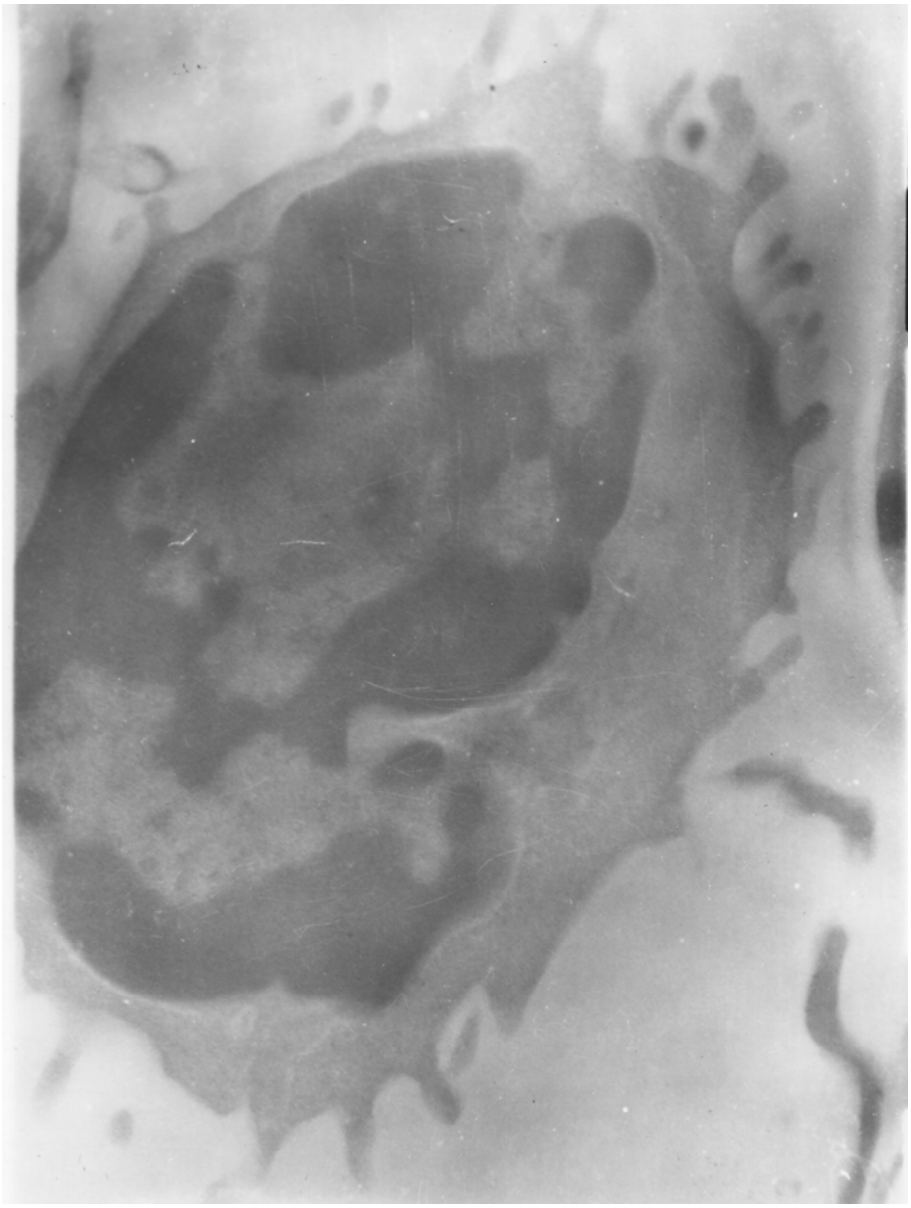

Fig. 10 A photo of a negative control slide exhibiting a cell free completely from positive immunoperoxidase reaction. X15,000

DISCUSSION

The major target organ for HBV is the liver. However, many genetic studies have suggested the infection of PBMCs with this virus (1-9). In the present work, a direct ultrastructural visualization of PBMCs isolated from patients with CHB established the presence of viral-like particles. However, the morphological appearance of such particles did not correspond exactly to the forms described in the infected hepatocytes in liver biopsy specimens or in the *in vitro* liver cell-line cultures (5, 13-22). In most of these ultrastructural studies , three forms were described. The double-shelled Dane body (42nm in diameter) represents the mature virus made of the nucleocapsid surrounded by an envelope and is localized in the ER cisternae of the hepatocyte cytoplasm. Another form is the subviral surface antigen spherical or filamentous particle (19-21nm in diameter) which represents an empty envelope free from the nucleocapsid core budding off the membranes of the ER cisternae and secreted into their lumen and then extracellularly (through fused ER and cell membranes). The ring-shaped core particle (20-25nm in diameter) was also described in the literature and represented the nucleocapsid situated in both the nucleus and

cytosol. The proposed function of the core particle was to recycle the daughter viral genome into the hepatocyte nucleus to amplify the pool of cccDNA in the replication cycle of the HBV (24).

In our research, particles resembling Dane bodies were not detected, but a possible counterpart was seen in the intracytoplasmic cisternae appearing without the double shell, bigger in size (100-120nm), homogeneously electron-dense and positively-stained for HBsAg. This could be viewed as a modified form of the Dane particle with variation in size and appearance owing to its presence in a different habitat with a possible incidence of mutation. In general, mutations in the viral genome were found to result in malformed particles e.g. in one study (25), the mutation in the Pre-S region led to a fried-egg-like shape of the complete virion and screw-like pattern of HBsAg filaments. Additionally, groups of small spherical rings (25-33nm in diameter) were detected in large intracytoplasmic cisternae resembling the spherical HBsAg subviral forms. None of these bodies were seen shedded extracellularly. Filamentous forms could not be detected. In fact, subviral filamentous forms were suggested to be formed by tubular budding of the membrane of the SER into the cisternal lumen (20), a phenomenon which is possibly restricted to hepatocytes and hence explaining their absence in PBMCs. Also, core particles could not be demonstrated in PBMCs of our study. This actually needs to be confirmed by immunostaining of PBMCs by anti-HBcAg antibody. It seems that recycling of the viral genome by the core particle into the nucleus is not a necessary event in the viral life cycle in PBMCs.

Generally, it is unclear whether the virion induces injury to infected extrahepatic tissues. As regards the liver, ultrastructural alterations of hepatocytes were disclosed by previous studies (20, 26-28) at the nuclear and cytoplasmic levels. The nuclei exhibited variable size, irregular shape, numerous enlarged cell nuclei and chromatin dissociation. The cytoplasm showed mitochondrial swelling, dilatation of ER and Golgi bodies and increase of lysosomes. In this study, cytopathic morphological features were also evidenced in the PBMCs but with more preservation of the nucleus. The infected cells, especially those containing the virion particles, displayed degenerative changes entailing widely dilated ER cisternae in disintegrated cytoplasm. Also some cells showed ultrastructural apoptotic changes. Therefore our study provides an evidence for a direct gross injurious effect of the intracellular virus on their host cells. This might explain apoptosis and impairment of killer activity and interferon-gamma responses of circulating lymphocytes induced by persistent HBV infection demonstrated in some studies (29-31).

Our findings point to variation in the mode of replication and morphogenic phases participating in the viral life cycle in the hepatocytes versus PBMCs. This could be due to the fact that hepatocytes constitute the specific normal habitat for the virus as they possess the various enhancers and regulators involved in the complete viral replicative cycle. On the other hand, PBMCs could serve as a reservoir for the latent virus with less replication competence and less ability to be discharged extracellularly. This view is supported by absence of intranuclear core particles, retention of the virus in the cisternae of ER , non-fusion of membranes of ER with the cell membrane and sparcity of positive deposits of HBsAg on the cell membranes of the labeled PBMCs.

On the basis of these findings and according to some details now known about replication strategy of HBV in hepatocytes (24, 32), a proposed comparative summary of the viral life cycles in both hepatocytes and PBMCs could be recapitulated. In hepatocytes, upon infection, the virus enters a cell and is transported to the nucleus, with concomitant uncoating. During this process, viral DNA is processed and converted to cccDNA. CCCDNA serves as the template for transcription of the major viral RNA including the pregenome. Some pregenomic RNA is subsequently packaged into viral cores and reverse transcribed to form relaxed circular DNA. Cores containing DNA may bud off the ER cisternae to be secreted as mature viral particles or may recycle directly to the nucleus to amplify DNA. In PBMCs, on the other hand, the initial steps of replication is similar to those occurring in hepatocytes until budding off of the viral cores into the lumina of ER cisternae and transformation into the mature complete virion. However, these virions remain

unshedded in a latent status in cytoplasmic cisternae together with the subviral surface antigen particles. Apparently, no recycling of the viral cores to the nucleus takes place and alternatively the virus may remain quiescent and hidden from the host's immune defence mechanisms (viral escape). When full disintegration of the cell occurs, the latent mature forms may then be released to reinfect other cells. This may result in persistence and chronicity of infection.

In conclusion, this study has shed new light on the various morphological phases of HBV in PBMCs, thus aiding in the understanding of the viral life cycle in the light of information gained from previous genetic studies. However, a long route of molecular and morphological investigations is still warranted on circulating and *in vitro* cultured mononuclear cells to follow the series of events of this extrahepatic replicative cycle during HBV infection. This may help to elucidate the role of extrahepatic cycle in affecting the prognosis, clinical outcome and antiviral therapeutic strategy of the disease.

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