Acta virol. **24** : 367—370, 1980

POLYPEPTIDE ANALYSIS OF HEPATITIS VIRUS TYPE B SURFACE ANTIGEN PRODUCED BY A HUMAN HEPATOMA CELL LINE, PLC/PRF/5

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Received April 16, 1980

Summary. — HBs antigen purified from culture fluid of a human hepatoma cell line, PLC/PRF/5, was analyzed by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis. At least 6 polypeptide species were resolved. The polypeptide with a molecular weight of 48,000 was identified as a glycoprotein.

Key words: *HBs antigen*; *polypeptide composition*; *polyacrylamide gel electrophoresis*

Attempts to propagate hepatitis B virus (HBV) in tissue culture have met with only limited success thus far (Zuckerman, 1975), which has greatly impeded the elucidation of intracellular events following HBV infection, and limited the extent of virological study of HBV itself. Such a limitation could be overcome, at least in part, by establishing a continuously growing human hepatic cell culture which carries the HBV genome, expresses viral functions and preferably produces virus particles.

A human hepatoma cell line, PLC/PRF/5, established by Alexander and co-workers (Macnab *et al.*, 1976) is of particular interest in this respect. The cell line, derived from the liver of an HBV surface antigen- (HBs Ag-) positive patient with primary hepatoma, is continuously excreting HBs antigen (subtype ad) into culture medium (Macnab *et al.*, 1976). At least most, and probably all, of the DNA sequence of the HBV genome is reportedly integrated in the cell chromosome (Marion *et al.*, 1980), but only HBs Ag is expressed, and neither HBV core antigen (HBc Ag) nor DNA polymerase activity is detectable in the culture. Virus particle is not produced even after an induction with iododeoxyuridine, aminopterin and hypoxanthine (Alexander *et al.*, 1978).

In the present experiment we analyzed the polypeptides of HBs antigen purified from culture fluid of PLC/PRF/5 cells by polyacrylamide gel electrophoresis.

Clone PLC/PRF/5 was a generous gift of Dr. J. Alexander. Since the original cell stock was found to be contaminated with *Mycoplasma orale* (type 1), we first passaged the cells in the presence of Josamycin, Lincomycin, Tylosine and type-specific antiserum to remove the contaminant from the culture. Such a treatment increased the amount of HBs antigen produced by the cells by 4- to 8-fold, and culture fluid with a reverse passive hemagglutination (R-PHA) titer of 1 : 32 to 1 : 64 was regularly obtained (Sugiura and Tobita, unpublished observation). Even after the cell sheet became confluent, the cells still continued to excrete the antigen at the same rate as when they were actively growing.

The cells were grown in Eagle's minimal essential medium containing 10% fetal calf serum (FCS) supplemented with non-essential amino acid mixture, and passaged at 3 days' intervals

at 1 : 3 split in 75 cm^2 plastic tissue culture flasks (Falcon). To obtain HBs antigen from culture fluid, cells were seeded to 150 cm^2 tissue culture flasks (Corning) and incubated at 37° C. When the cell sheet became confluent, dexamethazone (10 μg/ml) was added and the cultures were incubated at 34° C for an additional 4 days.

HBs antigen was purified from the culture fluid by 3 cycles of velocity sedimentation through a 5—30% linear sucrose gradient in NTE buffer (0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl, pH 7.4) at 35,000 rev/min for 4 hr in the Spinco SW 40 rotor at 4° C, followed by isopycnic banding in CsCl. At each purification step, the gradient was monitored for proteins and HBs antigen by reading the optic density at 280 nm and by R-PHA assay of the fractions collected from the bottom of the gradient. Those fractions which showed the highest R-PHA titer were harvested. HBs was finally pelleted at 48,000 rev/min for 4 hr at 4° C in the Hitachi RPS 50 rotor and suspended in twice distilled water.

HBs antigen thus purified was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The antigen was denatured by boiling for 2 min with 1% each of SDS and 2-mercaptoethanol (2-ME), and applied to a vertical polyacrylamide slab gel as described by Laemli (1970). The gel was 13% polyacrylamide (acrylamide/bis-acrylamide ratio of 26/0.2) containing 6 M urea and 0.1% SDS. The electrophoresis was performed at 40 V (constant voltage) for 17 hr at 20° C. After the run, the gel was stained with Coomassie brilliant blue or periodic acid — Schiff (PAS) reagent.

The results are shown in Fig. 1. HBs antigen was resolved into 7 polypeptide species (VP 1 to VP 7). The molecular weight of each polypeptide as estimated by using bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsin, soybean trypsin inhibitor and cytochrome C as marker proteins was; VP 1 — 12,000, VP 2 — 21,000, VP 3 — 23,000, VP 4 — 37,000, VP 5 — 43,000, VP 6 — 48,000 and VP 7 — 68,000. The general electrophoretic profile was consistent with that already described by others. Several points are worthy of particular mention.

First, it has been suggested that a polypeptide with a molecular weight of 68,000 (our VP 7) might reflect a mere contaminant of serum albumin (Marion *et al.*, 1979). In the current experiment, by reducing the antigen with 0.2 M dithiothreitol (DTT) and 2% SDS, VP 7 in Fig. 1 was resolved into 2 polypeptide species (7a and 7b, Fig. 2). VP 7b co-migrated with bovine serum albumin (BSA) similarly treated, while 7a migrated differently (Fig. 3). Therefore, it is unlikely that VP 7 totally reflects serum albumin, although it is still possible that 7b might be bovine serum albumin which had been carried over from the culture medium.

Second, VP 6 but not VP 3, was stained with PAS partly in agreement with the findings on HBs antigen in human serum (Wai-Kuo Shih and Gerin, 1977*a*), but in contrast with the findings on the antigen of the hepatoma cell culture origin (Marion *et al.*, 1979). Addition of dexamethazone in our culture system might have altered the mode of glycosylation of HBs antigen within the cells.

Third, if the antigen was boiled for 5 min or more with 0.2 M DTT and 2% SDS, the molecular size of VP 2 became smaller (Fig. 2). In addition, the lower half of the VP 3 band became more prominent as the sample was boiled longer (Fig. 2). These findings, together with the appearance of VP 7a after reduction with DTT, might indicate that some of the disulfide bonds were still maintained after 2-ME treatment within the polypeptides of HBs

antigen, which were cleaved under a more severe reducing condition, with subsequent loss of a small polypeptide piece.

VP 1 migrated at the same position as cytochrome C (Fig. 3). In the present experiment we could not exclude the possibility that this may actually have been cytochrome C of cellular origin (Marion *et al.*, 1979).

A basic question concerning the polypeptide composition of HBs antigen is how DNA of HBV with at most 4,900 base pairs (Landers *et al.*, 1977) exhibits such a large coding capacity. A hypothesis presented by Monjardino and Crawford (1979) is attractive in this respect. They suggested that HBs may consist of essentially a single polypeptide species with a molecular weight of 22,000, and all other HBs antigen-related polypeptides might be polymers of this single polypeptide. A close relatedness between two of the polypeptide species of HBs in amino acid composition (Wai-Kuo Shih and Gerin, 1977*b*) may support this view. In our hands, extensive reduction of the antigen did not give rise to a single polypeptide species as reported by Monjardino and Crawford (1979), although a modification of some of the polypeptides in terms of migration distance was clearly observed. Peptide mapping may be helpful in further elucidating the strategy of HBV.

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Explanation of Figures (Plate XXXI):

Fig. 1. SDS-PAGE of HBs antigen purified from culture fluid of PLC/PRF/5 cells. The antigen was boiled for 2 min with 1% each of SDS and 2-ME and subjected to electrophoresis in a vertical slab gel as is described in the text. The gel was stained with either Coomassie brilliant blue (I) or PAS reagent (II).

Fig. 2. Reduction of HBs antigen with DTT for different periods of time. The antigen was

boiled for 1 (I) 5 (II) or 10 (III) min in the presence of 0.2 M DTT and 2% SDS and subjected to PAGE. The gel was stained with Coomassie brilliant blue.

Fig. 3. Parallel electrophoresis of HBs antigen (I), bovine serum albumin (BSA — II) and cytochrome C (III). The purified HBs antigen was boiled for 1 min in the presence of 0.2 M DTT and 2% SDS. BSA and cytochrome C were similarly treated. The samples were run in parallel in 13% polyacrylamide gel for 16 hr at 40 V. The gel was stained with Coomassie brilliant blue.