NOTES

PLC/PRF/5 (Alexander) Hepatoma Cell Line: Further Characterization and Studies of Infectivity

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The Alexander hepatoma cell line, PLC/PRF/5, was studied for evidence of hepatitis B virus markers and alpha-fetoprotein. Only hepatitis B surface antigen and alpha-fetoprotein were detected. Induction experiments with 5-iodo-2'-deoxyuridine and inoculation of chimpanzees with whole cells or tissue culture fluid did not reveal evidence of synthesis of additional hepatitis B virus markers or of production of infectious virus.

Many important virological experiments with hepatitis B virus (HBV) cannot now be performed because this virus has not been grown *in vitro*. Although a number of reports of primary isolation have appeared, to date, serial passage of the virus has not been achieved (6, 14, 20, 21).

In 1976 the Alexander hepatoma cell line (PLC/PRF/5), which continuously produces hepatitis B surface antigen (HBsAg), was established (3, 9). Recently, a second hepatoma-derived cell line producing HBsAg was established (2). The PLC/PRF/5 cell line, which has been more extensively studied, was derived from the liver of a patient with primary liver cell carcinoma who had circulating HBsAg in his blood. Radioimmunoassays of the supernatant fluids from the cell line showed that significant amounts of HBsAg were released into the culture medium. However, no other markers of HBV synthesis such as hepatitis B core antigen (HBcAg), Dane particles, hepatitis B e antigen (HBeAg), or core particles have been detected in the culture medium or within the cells (4, 15).

The present studies were undertaken to further characterize the Alexander hepatoma cell line with respect to both HBV markers and infectivity.

The cell cultures used in this study included the PLC/PRF/5 human hepatoma cell line originally isolated by one of us (J.J.A.), the Mahlavu hepatoma-derived cell line, supplied by Patricia Marion of Stanford University, and African green monkey kidney (AGMK) cells obtained from Microbiological Associates, Rockville, Md. Cells were grown on cover slips in Leighton

tubes or in 150-cm² flasks. The growth medium was Eagle minimum essential medium with Earle salts and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer containing 10% inactivated fetal bovine serum (GIBCO, Long Island, N.Y.), 50 µg of gentamicin (Microbiological Associates) per ml, 2 µg of amphotericin B (Flow Laboratories, Rockville, Md.) per ml, and 2 mM glutamine (GIBCO). Cultures were grown in 5% CO₂ in air and incubated at 37°C. Maintenance medium was Eagle minimum essential medium containing 2% fetal bovine serum.

Cell cultures were examined for mycoplasma contamination, using methods that combined direct cultural procedures with indirect staining techniques applied to an indicator cell line (5, 12). Mycoplasmas recovered in culture were specifically identified, utilizing an agar plate fluorescent-antibody technique (7) and type-specific antisera. Mycoplasmas recovered as cell culture contaminants were also tested for their sensitivity (disk method) to chloramphenicol, gentamicin, and tetracycline. Cultures contaminated with mycoplasmas were treated with antibiotics to diminish the titer of mycoplasma (19) and were then additionally treated with specific mycoplasma antisera and guinea pig complement.

For immunofluorescence (IF), the cover slips were washed in phosphate-buffered saline (pH 7.4) and fixed in acetone. Direct IF was performed in a manner similar to that of Mathiesen et al. (11), except that a Xenon XB0 75 W or HB0 200 W mercury light source was used with

a Zeiss Universal fluorescence microscope. Photographs were made with a Zeiss 35-mm camera and Kodak EPT 135-36 film. The film was processed by the Kodak ESP-1 method. Fluorescein isothiocyanate-conjugated guinea pig hyperimmune antibody to HBsAg (anti-HBs) (13), human antibody to HBcAg (anti-HBc) (13), and chimpanzee hyperimmune antibody to hepatitis A virus (12) antisera were used for staining.

To confirm specificity of the IF reaction, blocking tests were performed with purified HBsAg (protein content, 241 $\mu\text{g}/\text{ml}$), normal guinea pig serum (GIBCO), and hyperimmune guinea pig anti-HBs (13). Blocking experiments were performed by preincubating the cover slips for 15 min with a 1:5 dilution of purified HBsAg, hyperimmune guinea pig serum, or normal guinea pig serum. The slides were then stained with the fluorescein isothiocyanate-labeled anti-HBs as described above.

HBsAg, HBeAg, anti-HBs, and anti-HBc were detected with commercially available radioimmunoassay methods developed by Abbott Laboratories, Chicago, Ill.: AUSRIA II for HBsAg, Ausab for anti-HBs, Corab for anti-HBc, and Abbott-HBe for HBeAg. HBsAg was quantitated by comparing values with a standard curve obtained with known concentrations of HBsAg. Cell culture medium, cell pellets, and chimpanzee sera were assayed for alpha-fetoprotein (AFP) by solid-phase radioimmunoassay (Phadebas AFP PRIST; Pharmacia, Uppsala, Sweden).

The thymidine analog 5-iodo-2'-deoxyuridine (Sigma Chemical Co., St. Louis, Mo.) was employed in attempts to induce complete virus synthesis. Stock solutions containing 10, 50, or 100 $\mu\text{g}/\text{ml}$ in tissue culture medium were prepared and stored frozen at -70°C until used.

After base-line liver function tests and liver biopsy, PLC/PRF/5 hepatoma cells or filtered supernatant fluids from the hepatoma cells were inoculated intravenously into seronegative chimpanzees. After inoculation, chimpanzees were tested weekly for HBsAg, anti-HBs, and AFP. Assays were performed for serum alanine aminotransferase, serum aspartate aminotransferase, and iso-citrate dehydrogenase.

Preliminary examination of the PLC/PRF/5 cell line (passage 18) on mycoplasma agar plates indicated that it was heavily contaminated. The major contaminant (estimated at about 94% of

mycoplasma population) was *Mycoplasma orale*; other species identified were *Mycoplasma hominis* (about 3%), *Mycoplasma bovis* (1%), *Mycoplasma alkalescens* (1%), and an unclassified bovine mycoplasma (70-159) (1%). Subsequent treatment of the cells with antibiotics and specific antiserum eliminated all except the *M. orale* strain.

When stained with anti-HBs, fluorescence was observed in over 90% of the cells by 7 days. HBcAg and hepatitis A virus antigen were not detected by IF. The HBsAg fluorescence was largely perinuclear but occasionally filled the cytoplasm, and occasional focal fluorescence was observed (Fig. 1). In a small number of cells, the IF staining had a coarsely granular appearance (Fig. 2). In addition, the HBsAg concentration appeared to increase with time as indicated by a greater intensity of staining with increasing age of the culture (Fig. 3). Both types of fluorescence were observed over a period of 10 cell passages.

To confirm the specificity of the IF reaction, blocking experiments were performed. Preincubation of the cells with purified HBsAg completely blocked all fluorescence. Normal guinea pig serum and phosphate-buffered saline were not effective in blocking, but hyperimmune guinea pig anti-HBs also blocked staining. Neither HBsAg nor HBcAg was detected by IF in the AGMK or Mahlavu cell lines.

Evidence that the PLC/PRF/5 cell line produced significant quantities of HBsAg and AFP was provided by radioimmunoassay of the supernatant tissue culture medium and cell pellets. A representative experiment is shown in Fig. 4. HBsAg was detected by day 5 in culture (0.01 $\mu\text{g}/\text{ml}$) and increased rapidly from day 8 to day 15, reaching a peak of $\geq 0.6 \mu\text{g}/\text{ml}$ at that time. In another experiment, a 15 $\times$ cell concentrate from a 3-week harvest (frozen-thawed and sonicated) contained 0.2 μg of HBsAg per ml at the same time that the unconcentrated tissue culture fluid contained 0.5 μg of HBsAg per ml. Samples of the supernatant fluids and cell pellets from the PLC/PRF/5 cell line were also assayed for the presence of HBeAg, another marker of HBV replication. No HBeAg was detected in any of the samples collected over 15 days. Supernatants from the Mahlavu hepatoma-derived cell line and AGMK were consistently negative for HBsAg and HBeAg.

FIG. 1. HBsAg in PLC/PRF/5 cell line detected by IF with fluorescein isothiocyanate-labeled anti-HBs, showing perinuclear and cytoplasmic fluorescence; 14-day-old culture. Bar, 20 μm .

FIG. 2. HBsAg in PLC/PRF/5 cell line detected by IF with fluorescein isothiocyanate-labeled anti-HBs, showing fluorescent granules scattered throughout the cytoplasm; 14-day-old culture.

FIG. 3. HBsAg in PLC/PRF/5 cell line detected by IF with fluorescein isothiocyanate-labeled anti-HBs, showing increased intensity of staining; 21-day-old culture.

FIGS. 1-3.
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FIG. 4. Production of HBsAg (Δ) and AFP ($\blacktriangle$) by the PLC/PRF/5 cell line. Samples of medium were removed from a T-150 T-flask at daily intervals. All samples were assayed in one test.

In contrast to previous studies (2, 3), AFP was detected in the supernatant throughout the growth period of the cell culture, reaching a peak of 86 ng/ml on day 11. Production of AFP paralleled that of HBsAg, but appeared sooner. A small amount of AFP was detected in the cell pellet (2.5 ng/ml). Control AGMK and Mahlavu cell cultures did not produce detectable AFP.

Certain thymidine base analogs, such as 5-iodo-2'-deoxyuridine, have been shown to induce cells to produce type C retroviruses (1, 8) and to enhance production of adenovirus (16) and cytomegalovirus (17, 18) in cells in which they do not replicate efficiently. Hepatoma cells were treated with three different 5-iodo-2'-deoxyuridine concentrations; 10, 50, or 100 μ g/ml was added to the medium at the time of planting and left in contact with the cells until they were harvested 7 days later. While remaining positive for the production of HBsAg, the cells did not produce detectable HBcAg by IF.

To determine whether the Alexander hepatoma cell line contains or synthesizes infectious HBV, two chimpanzees were inoculated with, respectively, hepatoma cells or cell-free medium from the cell cultures. A single-cell suspension (passage 30, 2-week-old culture) was prepared by removing monolayered cells from four T-150 flasks with Versene (ethylenediaminetetraacetic acid), 0.2 mg/ml (Microbiological Associates). The cells were pooled, washed with Hanks balanced salt solution to remove residual growth medium, and suspended in tissue culture medium with 5% autologous normal chimpanzee serum. Cell viability was $\geq 95\%$ as determined by trypan blue dye exclusion. IF of a smear of cells

used for inoculation showed the cells to be positive for production of HBsAg. A total of 15×10^6 cells in 7 ml of medium were infused intravenously into one chimpanzee. Twenty-five milliliters of the cell medium that had been added to the cultures on day 10 and harvested on day 14 was filtered through a 450-nm filter and infused into a second chimpanzee.

During the 7-month course of this experiment, weekly enzyme assays were performed. Levels of alanine aminotransferase, aspartate aminotransferase, and iso-citrate dehydrogenase remained normal. HBsAg was not detected in the serum. In addition, neither anti-HBs nor anti-HBc developed during this time. Neither chimpanzee developed elevated levels of AFP (all samples contained < 9 ng of AFP per ml), an indication that the hepatoma cells had not replicated significantly.

The continued production of HBsAg as observed by IF is consistent with the finding of Marion et al. (10), who found the complete viral genome to be present in the PLC/PRF/5 cell line. However, the failure to detect other HBV markers (HBcAg, HBeAg) or to transmit HBV to chimpanzees confirms the inability of the cell line to produce infectious virus particles.

The results of our experiments suggest that the PLC/PRF/5 cell lines does not represent a health risk to laboratory workers, since it does not produce detectable infectious virus. Careful monitoring, however, should be maintained, lest spontaneous production of virus should occur.

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