Tumorigenicity in Nude Mice of a Human Hepatoma Cell Line Containing Hepatitis B Virus DNA¹

Daniel Shouval,² Lola M. Reid,³ Prasanta R. Chakraborty, Nelson Ruiz-Opazo, Rachel Morecki, Michael A. Gerber,⁴ Swan N. Thung, and David A. Shafritz

Liver Research Center [D. S., L. M. R., P. R. C., N. R.-O., R. M., D. A. S.] and Departments of Molecular Pharmacology [L. M. R.], Pathology [R. M.], and Cell Biology [D. A. S.], Albert Einstein College of Medicine, Bronx, New York 10461, and Department of Pathology [M. A. G., S. N. T.], Mount Sinai School of Medicine, New York, New York 10029

ABSTRACT

The human hepatocellular carcinoma cell line PLC/PRF/5, which synthesizes and secretes hepatitis B surface antigen, was grown under optimal conditions in tissue culture, using Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 10⁻¹¹ m triiodothyronine on collagen rafts. Injection s.c. of the PLC/PRF/5 cell line into athymic BALB/c nude mice resulted in the growth of a well-circumscribed, moderately differentiated hepatocellular carcinoma. The intervals until tumor appearance and tumor "take" rates were dependent on inoculum dose. Four to 5 x 10⁶ cells induced tumor growth in 29% of 14 injected mice within 29 to 40 days, while 7 to 13 x 10⁵ cells induced tumors in all 15 mice within 10 to 12 days after inoculation. Hepatitis B surface antigen was detected in the nude mouse serum and tumor tissue, and its concentration roughly correlated with tumor weight. A low level of antibody against hepatitis B surface antigen was detected in five tumor-bearing animals, as well as in one mouse which did not produce a tumor. Hepatitis B core antigen and its antibody and hepatitis B e antigen and its antibody were not detected in 26 mice, using immunohistochemical and radioimmunoassay methods. α-Fetoprotein, carcinoembryonic antigen, and α-antitrypsin were detected in nude mice tumors, using the peroxidase-antiperoxidase technique. Finally, hepatitis B virus DNA, identified in the nude mouse tumor by molecular hybridization techniques, was compared to PLC/PRF/5 cell line hepatitis B virus DNA.

INTRODUCTION

Epidemiological evidence suggests an etiological relationship between HBV⁵ infection and PHC (10, 33). PHC is relatively frequent (25 to 100 cases/100,000 deaths) in China, Southeast Asia, sub-Saharan Africa, the Mediterranean Basin, and the Middle East, where HBV is endemic and 75 to 95% of patients with PHC have serological markers for HBV (10, 33). However, no direct evidence has been reported to show that HBV is oncogenic. In 1973, Alexander et al. (1, 2, 18) established in tissue culture a cell line PLC/PRF/5 from a hepatocellular carcinoma of a Mozambican male whose serum was positive for HBsAg. This cell line produces and secretes HBsAg into the growth medium. No other known HBV proteins or infectious virions (Dane particles) have been observed in PLC/PRF/5 cells or in the growth medium (2).

Congenitally athymic nude mice are used routinely as a host for various allogenic and xenogenic transplants as well as for tumorigenicity studies (8). Desmyter et al. (7) injected PLC/PRF/5 cells into BALB/c athymic nude mice and observed tumor formation at an inoculum above 5 x 10⁵ cells. Recently, we and others (5, 20) have shown by molecular hybridization with a specific ³²P-labeled HBV DNA probe that the PLC/PRF/5 cell line contains HBV DNA sequences integrated into the host genome and has distinct hybridizable RNA's (5). By characterizing PLC/PRF/5 tumors in nude mice, a model system for in vivo analysis of human hepatoma cells containing HBV DNA sequences can be established. Such a model could prove useful in studies of HBV, its relationship to hepatocellular carcinoma, and its interaction with the host. The present study was aimed to describe and compare properties of the PLC-PRF/5 cell line in vitro and tumors produced from these cells in vivo in BALB/c nude mice. These systems were used to assess tumorigenicity by inoculum size and to test the effect of the athymic host on HBV protein expression. Finally, HBV DNA was identified in the nude mouse tumor, and its restriction endonuclease digestion pattern was compared to that obtained from hepatoma cell line DNA.

MATERIALS AND METHODS

Culture Conditions. PLC/PRF/5 cells were cultured in T-75 or T-150 flasks (Falcon Plastics, San Francisco, Calif.) or 850-sq cm roller bottles (Corning Glassworks, Corning, N. Y.). All other plasticware was from Falcon. Cells were grown in MEM supplemented with L-glutamine, 10% heat-inactivated FBS, nonessential amino acids (10 mm), penicillin and streptomycin (100 µg/ml each), and Fungizone (2.5 µg/ml). All media components were obtained from Grand Island Biological Co. (Grand Island, N. Y.). T₃ was purchased from Sigma Chemical Co. (St. Louis, Mo.). Collagen gels were prepared by methods described previously (28).

Plating and Attachment Efficiency. Plating efficiency of the cells was ascertained by seeding 100 cells per 60-mm Falcon tissue culture dish, allowing clones to grow for 3 weeks fol-
lowed by fixation with methanol and staining with Van Gieson's stain. Clones were counted, and the plating efficiency was determined as the number of clones divided by the seeding density \( \times 100 \). The attachment efficiency was ascertained by seeding \( 10^6 \) to \( 10^9 \) cells/60-mm Falcon tissue culture dish. Twenty-four hr later, the medium was removed, the plates were rinsed with phosphate-buffered saline (Na\(_2\)HPO\(_4\), 8 mm; KH\(_2\)PO\(_4\), 1.5 mm; KC\(_i\), 2.4 mm; NaCl, 140 mm; phenol red, 5 mg/liter), and the cells were detached from the plate with trypsin (0.1%) plus EDTA (0.1 m). Cells were counted with a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.), and viability was determined by trypan blue exclusion. Attachment efficiency was calculated as the number of cells attached divided by the number of cells seeded \( \times 100 \).

**Growth Curves.** Cells were seeded at \( 10^5 \) on 60-mm Falcon tissue culture dishes. One of several media was added to a volume of 5 ml. The cells were incubated at \( 37^\circ \)C with 5% CO\(_2\)-95% air. The medium was changed daily. Cell counts from triplicate plates were determined on Day 1 and at varying time points thereafter, as specified in Tables 1 and 3 and Chart 1. At the time of each count, the medium was removed, the plates were rinsed with phosphate-buffered saline, and the cells were enzymatically removed (trypsin plus EDTA). Cells were counted with a Coulter counter, and viability was determined by trypan blue exclusion.

**Mice.** BALB/c nude mice obtained from G. Sato (University of California at San Diego, La Jolla, Calif.) were bred into a large colony (1200 to 1500) and maintained under strict isolation conditions. The colony is screened every 6 months for viruses, parasites, and other pathogens by serological assays and by autopsy of randomly selected experimental and breeder mice. Additional details regarding maintenance and isolation of animals were described previously (8). The normal life span of mice in this colony exceeds 18 months. For all experiments, 4- to 6-week-old mice were used.

**Preparation of Cells.** A subculture of human hepatocellular carcinoma cell line PLC/PRF/5 was obtained from Dr. I. Millman (Fox Chase Cancer Center, Philadelphia, Pa.). This cell line was derived from cultures established by Alexander et al. (1, 2). Cells were routinely screened for Mycoplasma by the technique of Chen (6) and were found to be negative. For infection, cells were washed and trypsinized, and viable cell number was determined by trypan blue exclusion. The cells were resuspended in 0.2 to 0.3 ml of serum-free MEM in a 1-ml tuberculin syringe and injected s.c. with a 23-gauge needle into the back of the nude mouse. Serial transplantation of tumors from mouse to mouse was performed after pressing tumors through a Collector (Bélico, Inc., Vineland, N.J.) and resuspending the fragments in serum-free medium.

**Morphological Studies.** Confluent monolayers of cells in culture and tumors from nude mice were fixed in 10% buffered formaldehyde and processed for paraffin embedding. Six-\( \mu \)m sections were stained with hematoxylin and eosin. All mice were autopsied at the termination of various experiments. Samples from nude mouse liver, spleen, lymph nodes, bone marrow, lungs, and kidneys were prepared for histology to ascertain host reaction to the tumor cells, the presence of metastases, and the presence of HBV markers.

**Immunohistochemical Studies.** Formalin-fixed, paraffin-embedded tissues from 4 tumors and 3 livers were used. Sections were deparaffinized and tested for the presence of HBsAg, HBcAg, \( \alpha \)-fetoprotein, carcinoembryonic antigen, and \( \alpha \)-antitrypsin by the peroxidase-antiperoxidase technique as reported previously (9, 35). The antisera and controls for specificity have been described (9).

**Serological Studies.** Plasma from nude mice was obtained by retrobulbar puncture in the second and fourth weeks after injection of tumor cells. Blood was collected in heparinized capillary tubes (Dade microhematocrit tubes) and centrifuged in an Adams microhematocrit centrifuge for 10 min. The capillary tubes were broken, and 30 to 80 \( \mu \)l of plasma were collected per mouse. Samples were pooled in groups of 2 to 5 and frozen at \(-20^\circ\). At the termination of the experiment, mice were anesthetized with 0.3 ml of tribromoethanol (Avertin; Winthrop, New York, N.Y.) i.p. and bled by cardiac puncture with a syringe containing 0.1 ml of sodium citrate per ml to prevent clotting. An average of 0.7 to 1.2 ml of whole blood were usually obtained. Two hundred to 500 \( \mu \)l of serum per mouse were recovered from recalculated blood and stored frozen at \(-20^\circ\) until use. Sodium azide (0.1%) was added to all tissue culture supernatants and recalculated plasma before freezing to prevent contamination.

**RIA for Detection of HBV Markers in Tissue Culture Medium and in Nude Mouse Serum.** Solid-phase RIA for detection of various HBV markers was performed with commercially available kits (Abbott Laboratories, North Chicago, Ill.) (25). HBsAg was determined by Ausria II-125 (16) and in several cases was repeated by reverse passive hemagglutination (Auscell) (13). All positive results for the first 10 tumor-bearing mice were confirmed by the Ausria II-125 neutralization kit. Anti-HBs was determined with an AUSAB kit (25); anti-HBe was determined with a CORAB kit (24). Polystyrene beads and reagents for RIA of HBeAg and anti-HBe (23) were a generous gift from Drs. L. R. Overby and I. K. Mushahwar (Diagnostic Division, Abbott Laboratories). For RIA tests, 200 \( \mu \)l of medium or serum were utilized for determination of HBsAg, anti-HBs, and HBeAg, while 100 \( \mu \)l were used for the detection of anti-HBe or anti-HBc.

Because of the small volumes of serum obtained from each mouse, we could not perform all 5 tests on the same animals. Therefore, sufficient animals were included in each experiment to permit separate groups to be used for each assay. In some cases, samples from the same group were pooled together for a given point. All RIA results are expressed as \( \times 10^8 \) cpm obtained in a \( \gamma \)-counter with automatic background subtraction (KLB Wallace, 1280 Ultra Gamma, Bromma, Sweden). Dilutions for quantitation of HBsAg in tissue culture medium were performed using fresh MEM supplemented with 10% FBS. Dilutions for HBeAg quantitation in mouse serum were performed with normal BALB/c nude mouse serum. Values for HBsAg production during cell growth (Chart 1) are expressed in cpm obtained from undiluted medium. Quantitation of HBsAg production in tissue culture and in nude mouse serum was performed using a positive control stock solution containing HBsAg at 20 ng/ml. This solution was diluted in 2-fold steps until the cpm of the negative control was reached. After the \( \times 10^4 \) cpm value was determined for each dilution point, a standard curve was drawn. All positive samples were serially diluted until the cpm reached a value 2.1 times above the mean negative control. This point was used to determine HBsAg concentration. All assays were performed from tissue culture medium in triplicate and from nude mouse serum in duplicate. Data are expressed as a range.
Identification of HBV Sequences in Cell Cultures and in Tumors from Nude Mice. HBV-DNA sequences were identified in DNA extracts from the PLC/PRF/5 cell line as reported previously (5, 30) and from PLC/PRF/5 tumors in athymic nude mice, using a $^{32}$P-labeled HBV-DNA probe (29). Briefly, 50 to 100 mg of tumor tissue (stored in liquid nitrogen) or 2 $\times$ 10^5 cells from confluent cultures were trypsinized and detergent lysed by the method of Innis and Miller (14). DNA was obtained by homogenization in 5 volumes of an equal mixture of phenol-1% sodium dodecyl sulfate; 50 mM Tris HCl, pH 7.5; 100 mM NaCl; and 2 mM EDTA followed by 2 phenol extractions and 2 ethanol precipitations. Total nucleic acids were resuspended in 50 mM Tris-HCl, pH 7.4-2 mM EDTA; 0.2 mM sodium acetate, pH 5.5, was added; and nucleic acids were reprecipitated with 3 volumes of ethanol at -20°. The final material was resuspended in 50 mM Tris-HCl, pH 7.4-100 mM NaCl, 2 mM EDTA. It was digested with pancreatic RNase, 25 to 50 $\mu$g/ml (recrystalized; Worthington, Freehold, N. J.), for 2 hr and treated with proteinase-K, 50 to 100 fig/ml, in the presence of 0.5% sodium dodecyl sulfate at 37° for 1.5 hr. This was followed by 2 phenol extractions and 2 ethanol precipitations. The final DNA pellet was washed with 70% ethanol, resuspended in appropriate buffer, and either applied directly to a 5-mm-thick, 0.8% agarose slab gel or digested 3 times with restriction enzymes as described in Fig. 2. All digestions with restriction endonuclease were performed in appropriate buffers at 37° for 6 to 12 hr. Twenty to 30 $\mu$g of each digested DNA sample were placed on a separate lane of a 0.8% agarose slab gel, and electrophoresis was performed as reported previously (5). After electrophoresis, DNA fragments were transferred to diazobenzoyloxymethyl cellulose paper (Schleicher and Schuell, Inc., Keene, N. H.), and the paper was hybridized with a “nick-translated” $^{32}$P-labeled pA01-HBV DNA probe (31). Nick translation was performed according to a modified procedure of Rigby et al. (29), using Escherichia coli DNA polymerase I and both $[^{32}P]$dATP and $[^{32}P]$dCTP (300 to 800 Ci/mmol) to give a specific activity of 2 x 10^6 cpm/ig DNA.

RESULTS

Characterization of PLC/PRF/5 Cells in Vitro. As shown in Fig. 1a, cells in confluent cultures of the PLC/PRF/5 line have a polygonal shape similar to that of human hepatocytes. The cells have prominent nuclei, multiple nucleoli, and granular appearance of the cytoplasm. During the first 24 to 48 hr after seeding, cells often appear vacuolated. With increasing cell density and time, vacuolization disappears. At high density, the cells often appear vacuolated. With increasing cell density and time, vacuolization disappears. At high density, the cells often appear vacuolated.

Table 1

<table>
<thead>
<tr>
<th>Medium</th>
<th>Serum</th>
<th>Substrate</th>
<th>Seeding density (no. of cells)</th>
<th>Plating efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>10% FBS</td>
<td>Plastic</td>
<td>$1 \times 10^2$</td>
<td>5.7</td>
</tr>
<tr>
<td>MEM</td>
<td>10% FBS</td>
<td>Collagen</td>
<td>$1 \times 10^2$</td>
<td>31.3</td>
</tr>
<tr>
<td>RPMI</td>
<td>10% FBS</td>
<td>Plastic</td>
<td>$1 \times 10^2$</td>
<td>13.0</td>
</tr>
<tr>
<td>RPMI</td>
<td>10% FBS</td>
<td>Collagen</td>
<td>$1 \times 10^2$</td>
<td>26.3</td>
</tr>
</tbody>
</table>

Higher seeding densities

<table>
<thead>
<tr>
<th>Medium</th>
<th>Serum</th>
<th>Substrate</th>
<th>Seeding density (no. of cells)</th>
<th>Attachment efficiency (%)</th>
<th>Doubling time (hr)</th>
<th>Saturation density (no. of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>10% FBS</td>
<td>RPMI</td>
<td>$1 \times 10^2$</td>
<td>70.0</td>
<td>9.5 x 10^5</td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$ RPMI, Roswell Park Memorial Institute Medium 1640; DME/F12, Dulbecco's modified Eagle's medium; Ham's F-12 (1:1).

$^{b}$ Lot A193219.

$^{c}$ Supplemented with $10^{-11}$ M T$_3$ (3).
lymph nodes, and kidneys was within normal limits. HBsAg and HBcAg were not detected in the liver of tumor-bearing mice by the peroxidase-antiperoxidase technique.

**Morphology of Tumors in Athymic Nude Mice.** Provided that inoculum density exceeded $4 \times 10^6$ cells, a small white s.c. nodule 10 mm in diameter appeared at the inoculation site after 4 to 5 days. Following initial appearance, nodules underwent transient regression (Fig. 1b). In most animals, tumors were not detected until Day 10 to 12, depending on the initial inoculum size (Table 2). After reappearance, nodules grew rapidly and acquired a brown-blush color, coinciding with macroscopic vascularization on the tumor surface. Twenty-eight to 42 days after inoculation, tumors were solid but later became friable and necrotic when tumor weight exceeded 1 to 2 g. At autopsy (1 to 6 months after inoculation), tumors were dissected easily from surrounding tissue and showed no invasion of deeper structures. Depending on inoculum density, tumor weight reached a peak of approximately 9 g after 6 months. Microscopic examination showed well-circumscribed single nodules encapsulated by fibrous tissue (Fig. 1c). Cell population was homogeneous and consisted of medium-size cells with abundant eosinophilic cytoplasm and nuclei with prominent nucleoli. Occasional multinucleated giant cells were present, and mitotic figures were numerous. Cells were arranged in masses, which sometimes surrounded and lined spaces containing an amorphous eosinophilic material or RBC (Fig. 1c). Some cells contained a foamy cytoplasm, while others contained inclusions. 'Ground-glass' cells were not identified.

There was no formation of bile. Necrosis of isolated cells gave rise to structures resembling 'eosinophilic bodies.' Larger tumors displayed areas of necrosis and hemorrhage. The general appearance was that of a moderately well-differentiated hepatocellular carcinoma.

**Detection of HBV Markers in Cell Cultures.** HBsAg production paralleled cell growth (Chart 1). Determination of HBsAg levels in medium by 2-fold serial dilutions indicated production of approximately 300 ng/$10^6$ cells/24 hr at confluence. HBsAg values given in Table 3 were from undiluted samples but, after an initial decrease, roughly paralleled cell growth. As shown in Table 3, varying medium conditions did not have a significant effect on HBsAg production. During the first 5 days in culture, variations in HBsAg levels were high. A significant rise in HBsAg was noted on the ninth day, which corresponded to the latter part of the logarithmic growth curve. The only parameter which seemed to influence HBsAg production was cell number during the fifth to ninth day after seeding. HBsAg was not detected in 10 different supernatants from confluent cultures with 6 to $8 \times 10^6$ cells each.

**Detection of HBV Markers in Nude Mice.** As shown in Table 2, HBsAg production also paralleled tumor growth in the nude mouse. HBsAg could be detected in the plasma within 2 to 4 weeks after inoculation when tumor diameter was still less than 10 mm. In the mouse, HBsAg could be detected in the plasma at 45 days after inoculation (Table 2). Mean negative control value for this assay was 290 ± 32 cpm (S.E.) in 7 normal BALB/c nude mice.

### Table 2

<table>
<thead>
<tr>
<th>Dose of injected cells ($10^6$)</th>
<th>Mice with neoplasms/ mice given injections</th>
<th>Time until tumor appearance (days)</th>
<th>Wt of neoplasm at 45 days (g)</th>
<th>Serum HBsAg ($10^{15}$ cpm range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–3</td>
<td>0/16</td>
<td>29–40</td>
<td>NT</td>
<td>145–380</td>
</tr>
<tr>
<td>4–5</td>
<td>4/14</td>
<td>12</td>
<td>0.4–1.1</td>
<td>2,240–5,905</td>
</tr>
<tr>
<td>7</td>
<td>5/5</td>
<td>12</td>
<td>2.9</td>
<td>2,890–14,328</td>
</tr>
<tr>
<td>10</td>
<td>9/9</td>
<td>10–12</td>
<td>1–3</td>
<td>3,080–49,383</td>
</tr>
<tr>
<td>13</td>
<td>0/1</td>
<td>10</td>
<td>2.9</td>
<td>NT</td>
</tr>
</tbody>
</table>

*Data were obtained at time of sacrifice on the 45th day. Mean negative control value for this assay was 290 ± 32 cpm (S.E.) in 7 normal BALB/c nude mice.

### Table 3

**HBsAg production under various culture conditions**

Cells were seeded onto 60-mm Falcon tissue culture dishes in triplicate. All media were supplemented with 10% FBS (Lot A193219). Cells were counted and medium was frozen at −20° at days indicated. Medium was replaced in the remaining uncounted dishes on Days 1 and 5 after seeding. Supernatants from 7 conditioned media, derived from a confluent Morris hepatoma cell line, were used as controls for HBsAg determination. Mean $10^{15}$ cpm of the negative control for the RIA was 395.

#### Mean no. of cells/HBsAg (mean $10^{15}$ cpm on various days after seeding)

<table>
<thead>
<tr>
<th>Medium</th>
<th>No. of cells</th>
<th>Day 1</th>
<th>Day 5</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>$1 \times 10^4$</td>
<td>$9 \times 10^6/159$</td>
<td>$8.5 \times 10^6/348$</td>
<td>$3.4 \times 10^6/815$</td>
</tr>
<tr>
<td>MEM</td>
<td>$1 \times 10^5$</td>
<td>$9.2 \times 10^6/1,607$</td>
<td>$1.4 \times 10^6/5,732$</td>
<td>$4.3 \times 10^6/13,483$</td>
</tr>
<tr>
<td>MEM + T5 ($10^{-11}$ M)</td>
<td>$1 \times 10^6$</td>
<td>$7.3 \times 10^6/1,407$</td>
<td>$5.7 \times 10^6/3,844$</td>
<td>$5.3 \times 10^6/15,548$</td>
</tr>
<tr>
<td>DME/F12</td>
<td>$1 \times 10^6$</td>
<td>$6.7 \times 10^6/795$</td>
<td>$8.2 \times 10^6/2,992$</td>
<td>$5.4 \times 10^6/13,417$</td>
</tr>
<tr>
<td>RPMI</td>
<td>$1 \times 10^6$</td>
<td>$7.0 \times 10^6/1,492$</td>
<td>$7.2 \times 10^6/2,403$</td>
<td>$8.0 \times 10^6/12,076$</td>
</tr>
</tbody>
</table>

*DME/F12, Dulbecco’s modified Eagle’s medium; Ham’s F-12 (1:1); RPMI, Roswell Park Memorial Institute Medium 1640.
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Table 4

All mice were given injections of 7 to 10 x 10^6 cells and sacrificed 4 to 8 weeks after inoculation. For detection of HBsAg, anti-HBs, and HBeAg, significant elevation above mean negative control represents a positive result. For anti-HBc and anti-HBe, significant reduction in counts would represent positive results. All assays were standardized against positive and negative HBV human sera provided with the commercial reagents.

### Sample HBV marker No. of mice 
Control HBsAg 14 2.41 ± 1.44
Experimental HBsAg 7 25.21 ± 11.936 <0.007
Control Anti-HBs 5 1.20 ± 6 NS
Experimental Anti-HBs 14 7.47 ± 244 NS
Control HBeAg 5 6.05 ± 15
Experimental HBeAg 11 777 ± 31 NS
Control Anti-HBe 7 4.606 ± 15
Experimental Anti-HBe 3 4.506 ± 254 NS
Control Anti-HBc 5 6.022 ± 274 NS
Experimental Anti-HBc 12 5.960 ± 419 NS

* Mean ± S.E.

NS, not significant. Although the mean anti-HBs level was elevated in 14 mice, the p value was not significant due to variations from mouse to mouse. However, in 5 mice, mean anti-HBs level was 1461 ± 316 cpm, which was significantly elevated as compared to controls (p < 0.003).

10 mm HBsAg was undetectable in mice when tumor weight was below 80 mg. HBsAg concentration exceeded 2500 ng/ml when tumor weight was 4 g. In 2 animals, HBsAg production appeared initially during a transient stage of white nodule formation but disappeared as the nodule regressed. Five of 14 mice with tumors had significantly elevated anti-HBs 5 to 8 weeks after injection of 7 to 10 x 10^6 cells (Table 1). A group of 8 mice given injections of 5 x 10^6 cells without development of tumors, one animal had a significant anti-HBs level (1655 cpm) 5 weeks after injection. HBeAg, anti-HBe, and anti-HBc were not detected in all animals studied.

**Immunohistochemical Staining.** Immunoperoxidase studies revealed HBsAg in more than 50% of tumor cells in 3 mice but rarely in tumors in a fourth animal (Fig. 1d). Reaction product was seen as fine granules throughout the cytoplasm of tumor cells. HBeAg was not detected. α-Fetoprotein and α1-antitrypsin were demonstrated as coarse granules in the cytoplasm in 30 to 50% of tumor cells in all cases. Carcinoembryonic antigen was detected primarily along the plasma membrane in 5 to 10% of tumor cells. The liver of 3 animals studied did not contain α1-antitrypsin or viral oncofetal antigens.

**HBV DNA Sequences in the PLC/PRF/5 Cell Line and in PLC/PRF/5 Tumors in Nude Mice.** DNA from the PLC/PRF/5 cell line and PLC/PRF/5 tumors was treated with restriction endonuclease EcoRI, which recognizes one site in the HBV genome. As shown in Fig. 2, there are 4 major HBV bands with both PLC/PRF/5 cell line DNA (Fig. 2, Lane A) and mouse tumor DNA (Fig. 2, Lane B). The EcoRI restriction enzyme patterns are the same except for one additional low-molecular-weight fragment (1000 to 1500 base pairs) present in the cell line DNA. The molecular weights of the radioactive bands ranged from 2100 to 4600 base pairs.

**DISCUSSION.**

The present study described the tumorigenicity of a human hepatocellular carcinoma cell line containing HBV DNA sequences (5, 20) in congenitally athymic BALB/c nude mice. The hepatoma cell line in culture and in the nude mouse provides us with 2 well-defined environments which can be used for investigation of the relationship between HBV infection and hepatocellular carcinoma. To date, HBV has not been grown as a lytic infection in tissue culture and has not been shown experimentally to induce hepatocellular carcinoma. A virus with properties similar to those of HBV, however, has been identified in woodchucks with hepatocellular carcinoma (32), and a similar virus has been observed recently in serum from ground squirrels (19).

Both tissue culture and nude mouse tumor growth of PLC/PRF/5 cells provide avenues for investigating HBV oncogenicity. Optimal growth conditions for PLC/PRF/5 cells in tissue culture were obtained using collagen gels as substrate with MEM supplemented with 10% FBS and 10^-11 M T3. Successful transplantation in the nude mouse was achieved at inoculum densities above 4 x 10^6 cells, and both tumor growth rate and final mass were increased at higher inoculum densities. PLC/PRF/5 cells in tissue culture demonstrate several differentiated hepatocyte functions, including production of specific hepatic proteins [transferrin, ceruloplasmin, α2-macroglobulins, and α1-antitrypsin (15)], and their morphological appearance resembles human hepatocytes. Oncofetal proteins such as α-fetoprotein and carcinoembryonic antigen are also produced and secreted in vitro (9) and in vivo in nude mice (present study).

The PLC/PRF/5 cell line and nude mouse tumor demonstrate some features of persistent viral infection as defined by Lwoff (17) and more recently by Holland et al. (12). The majority of the cells in vitro and in vivo produce HBsAg. The ability to secrete HBsAg is maintained as a stable phenotype during propagation in tissue culture. Twenty-four subclones of this line continue to synthesize HBsAg without isolation of a "nonproducer" to date. The requirement for a relatively high inoculum density of PLC/PRF/5 cells to yield tumors in nude mice suggests that tumorigenicity of the cell line may be suppressed by host control mechanisms. Previously, it was shown that persistent infection of HeLa cells or baby hamster kidney cells with mumps, measles, or influenza viruses causes a significant decrease in the tumorigenicity of cells in nude mice (21, 26) which has been correlated with augmented NK cell activity. Demonstration of the persistent viral state should also include evidence of occasional expression of infectious HBV. This may, in fact, have occurred in the periodic lysis of some cultures as described in "Results," and this is under further investigation. However, periodic cell crisis could also be explained by Mycoplasma contamination, which we did not detect in our cell stocks.

From molecular hybridization studies, it is evident that HBV DNA sequences are present in the cell line and in the nude mouse tumor. Using restriction enzyme digestion with endodeoxyribonuclease HindIII, which has no internal cleavage site within the HBV genome, it was demonstrated previously that HBV DNA sequences in the cell line are integrated into the host genome (5, 20). This is also true in the nude mouse tumor under present conditions where general fidelity of integrated HBV-DNA is maintained (Fig. 2). The absence of the low-molecular-weight hybridizable band in the tumor DNA is not understood at the present time, although it probably reflects...
dilution of HBV sequences in a solid tumor containing much stromal tissue versus the single-cell type as grown in tissue culture.

Among the various HBV proteins, only HBsAg was produced in vitro and in vivo in proportion to cell number or tumor mass. Immunohistochemical staining confirmed the presence of cytoplasmic and membranous HBsAg in the majority of cells. HBcAg and HBeAg were not detected in vitro (9) or in vivo. Antibodies against HBCAg and HBeAg were also undetectable in the serum of nude mice, regardless of whether tumor transplantation was successful or not. These data confirm previous observations obtained with less sensitive techniques. Although the various RIA’s are highly sensitive, there still is a possibility that some of the cells produce HBeAg or HBCAg below detectable levels. Low anti-HBs levels were detected in sera from nude mice with small tumors as well as in one mouse which did not have a tumor. This suggests that anti-HBs production may influence tumor “take.”

In conclusion, a model system has been described to study the propagation of a human hepatocellular carcinoma cell line in BALB/c nude mice. Many features of this system are similar to those reported in NMRI nude mice (7). PLC/PRF/5 cells are probably persistently infected with HBV. HBV DNA is integrated into the host cell genome in the cell line as well as in solid tumors produced in nude mice from this cell line. Only HBsAg is secreted into the nude mouse serum, and its concentration is proportional to cell mass. Transfer of the cell line to the nude mouse host did not cause expression of other HBV proteins (HbcAg and HBeAg) but did enable us to produce large quantities of tumor tissue. PLC/PRF/5 tumors in nude mice are well encapsulated with practically no invasiveness. The low tumorigenic potential of these virus-infected cells and the lack of invasive potential may be influenced by host factors. The nude mouse is not totally immunodeficient and has residual host defense mechanisms, such as NK cell activity (11). The NK system is known to reduce the tumorigenicity of virus-infected cells (21, 22, 24) and to suppress invasiveness and metastatic potential of tumor cells (27). Further studies on enhancement of tumorigenic potential of PLC/PRF/5 cells in nude mice will be presented elsewhere.

During final preparation of this manuscript, Bassendine et al. (4) reported successful growth of PLC/PRF/5 cells in athymic mice. Although some data presented in these studies are similar, there are fundamental differences regarding the dose of injected cells necessary to produce s.c. tumors. Bassendine et al. (4) reported a relatively low “take” rate of injected cells, although pretreatment of animals with irradiation augmented the take rate from 8 to 62% at 5 x 10^6 injected cells and from 38 to 75% at 1 x 10^7 cells. Increasing the inoculation dose and irradiation also reduced the interval until tumor appearance from 5 to 15 weeks to 3 weeks. Our data show that increasing inoculum dose improves take rate of tumors from 29% at 4 to 5 x 10^6 cells to 100% at 7 to 10 x 10^6 cells without pretreatment of mice with irradiation. The time interval until tumor appearance is also shorter (4 to 6 weeks at 4 to 5 x 10^6 cells and less than 2 weeks at 7 to 10^6 cells). These observations are in accordance with the preliminary report of Desmyter et al. (7) and may reflect differences in mice stocks, age of animals, preparation of cells, or variations in injection techniques. There may also be differences in NK cell activity in different stocks of mice.

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Fig. 1. Photomicrographs of PLC/PRF/5 cells in tissue culture and in s.c. tumors in BALB/c nude mice. a, monolayer of PLC/PRF/5 cells in culture. Phase microscopy, × 200. b, s.c. tumor in a nude mouse 12 days after inoculation of cells. The tumor is well circumscribed. The lower part of the tumor shows fibrosis and calcification. On the right upper portion, note viable tumor cells. H & E, × 96. c, microscopic appearance of a s.c. tumor in a nude mouse during the rapid growth phase. C, tumor capsule; S, secretory space; arrows, mitosis. H & E, × 150. d, localization of HBsAg in a nude mouse s.c. PLC/PRF/5 tumor by the peroxidase-antiperoxidase technique, using goat anti-HBs and counterstaining with hematoxylin. HBsAg is demonstrated as fine granules in the cytoplasm of many tumor cells (arrows). × 200.
Fig. 2. Autoradiogram of an 0.8% agarose gel of EcoRI-digested cell line DNA (Lane A) and nude mouse hepatoma DNA (Lane B). The base pair length of HBV DNA bands was determined by comparison to restriction fragments of plasmid pBR322 and cloned HBV DNA (data not shown). HBV DNA sequences were identified by molecular hybridization with a 32P-labeled pA01 HBV DNA probe (see "Materials and Methods" for experimental details).
Tumorigenicity in Nude Mice of a Human Hepatoma Cell Line Containing Hepatitis B Virus DNA

Daniel Shouval, Lola M. Reid, Prasanta R. Chakraborty, et al.


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