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A Human Hepatoma Cell Line (PLC/PRF/5) Produces Lung Metastases and Secretes HBsAg in Nude Mice*

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Abstract—*PLC/PRF/5 is a human liver cancer cell line which synthesizes hepatitis B virus surface antigen (HBsAg). These cells produced tumor in* 6 *of* 8 (75%) *congenitally athymic nude mice at* 13 *of* 28 *subcutaneous injection sites and in* 13 *of* 14 (93%) *mice inoculated intraperitoneally. Tumors were successfully transplanted to* 4 *of* 6 *additional nude mice. Tumor growth was rapid. Growth of cephalad tumors was significantly greater than for caudal tumors* (0.39 mm/day *versus* 0.28 mm/day). *Microscopic examination of tumors showed moderately well-differentiated hepatocellular carcinoma. Foci of identical cells were present in pulmonary veins in* 7 *of* 14 *tumor-bearing animals. Tumor cell karyotype was identical with that of PLC/PRF/5 cells. In addition, HBsAg was detectable in high titer in animals with extensive tumor. Biological features of PLC/PRF/5-induced tumors in nude mice appeared to closely resemble human hepatocellular carcinoma. Moreover, HBsAg may provide a marker of tumor growth.*

INTRODUCTION

HEPATOCELLULAR carcinoma is a leading cause of cancer-related deaths in Asia, Africa and Greece. In these areas hepatitis B virus (HBV) appears to be causally related, since active infection is found in up to 90% of cases, as evidenced by the presence of viral coat antigen (HBsAg) or antibody directed against the internal core (anti-HBc) of the virus in the serum of patients with tumor [1, 2]. Moreover, HBV antigens may be found in malignant hepatocytes as well as in the surrounding non-malignant liver cells [2, 3]. In control populations without primary liver cancer in the same countries, HBV infection is 5- to 10-fold less prevalent than in persons with primary liver cancer. The causal relationship is further strengthened by the fact that primary liver cancer cell lines (PLC/PRF/5 and Hep 3B) have been developed from two patients who had circulating HBsAg [4, 5]. Both cell lines secrete HBsAg into the supernatant [5, 6], but other HBV gene products such as cores, intact HBV (Dane) particles or virion-associated DNA polymerase activity have not been detected in these cells [7, 8]. Moreover, in both cell lines the entire HBV genome is integrated into hepatocyte DNA [7, 9], which simulates the transformed state associated with other oncogenic animal viruses such as Epstein–Barr virus and Simian virus 40.

Development of an animal model in which tumor growth occurs after transplantation of these cell lines would facilitate study of the biology of primary liver cancer associated with HBV. It is therefore of importance that these cell lines produced tumors when injected in nude mice [10, 11]. In one study tumors developed only when inoculated beneath the renal capsule [10]. In another study PLC/PRF/5 cells produced subcutaneous tumors at the site of inoculation in irradiated nude mice, though tumor growth occurred in only 4 of 27 (15%) non-irradiated animals [11]. At about the same time we produced tumors in nude mice by

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intraperitoneal inoculation of PLC/PRF/5 cells (F.R.S., S.P. and J.W.K., unpublished observations). We performed additional studies to characterize aspects of this model previously not described. This report describes the successful transplantation in high yield of PLC/PRF/5 tumor cells in non-irradiated nude mice by simple subcutaneous or intraperitoneal inoculation. It is the first description of pulmonary metastases in PLC/PRF/5 tumor-bearing animals, demonstration of regional difference in the growth rate of this tumor in nude mice, correlation of serum HBsAg levels with tumor size and documentation that the tumor cell karyotype is stable *in vitro* and is not altered by passage in mice.

MATERIALS AND METHODS

Tissue culture

A human hepatocellular carcinoma cell line, PLC/PRF/5 [4], derived from a 24-year-old male with primary liver cancer and whose blood contained HBsAg, was maintained in Dulbecco's modified Eagle's media supplemented with 7.5% fetal bovine serum, sodium bicarbonate and antibiotics under 5% CO_2 at 37°C. Cells for animal inoculation were harvested from passage number 116 by gentle trypsinization (0.05% trypsin and 0.001 mM EDTA). Concentrations of viable cells were determined by trypan blue exclusion.

Tumors were removed from mice by blunt dissection under sterile conditions. Tumor tissue was cut into 1-mm^3 fragments and dissociated for 20 min with DNAase and pronase in Eagle's basal media without calcium. The cells were washed three times, resuspended in minimal essential media supplemented with 7.5% fetal bovine serum, L-glutamine and sodium bicarbonate, and plated in plastic flasks.

Mice

Weanling congenitally athymic nude (nu/nu) mice [N:NIH(S) Webster, sixth backcross generation] were obtained from Life Sciences, Inc., St. Petersburg, FL. Mice were handled under stringent conditions to avoid infection and maintain optimal health. Groups of 5–6 animals were housed in plastic cages within a flexible film isolator equipped with HEPA air filtration. Initial sterilization of the isolator and all transfers through the docking port were done with aerosolized 2% peracetic acid.

Method of inoculation and measurement of tumor size

Four- to six-week-old mice were inoculated with 0.1 ml (8×10^5–10^7 cells) into each of 4 flank sites (2 caudad and 2 cephalad) or 0.5–1 ml (3×10^6–10^8 cells) intraperitoneally. Solid PLC/PRF/5 tumors excised from animals that developed tumor nodules at subcutaneous injection sites were cut into 1-mm^3 pieces and inoculated through a 13-gauge trocar into other nude mice.

Animals were examined daily for signs of illness and tumor growth. The latent period was defined as the first appearance of subcutaneous tumor growth measurable in at least two diameters. When tumors reached sufficient size, measurements were made in three dimensions, three times per week. Growth rates of subcutaneous tumors were determined from calculations of geometrical mean diameters ($\sqrt[3]{(L \times W \times H)}$). Intra-abdominal tumor growth was detected *pre mortem* by the appearance of opaque nodules which were readily seen through the transparent skin over the abdominal wall.

Chromosome analysis

At 48–72 hr after replating, cultures were treated with Colcemid (0.5 μg/ml) for 2–4 hr and harvested by gentle trypsinization (Gibco EDTA–trypsin combination). Cells were suspended in 0.075M KCl for 15 min at 37°C and fixed in freshly prepared cold methanol–acetic acid (3:1). Fixative was changed 3 times after 30-min intervals and cells were suspended in fresh fixative overnight. Cells were dispersed in fixative to achieve an even spread on cold, moist slides, air-dried for 30–40 sec and stored at 4°C. Metaphase spreads were evaluated by Q- and G-banding [12, 13].

Tests for HBsAg and DNA polymerase activity

One hundred to 150 μl of blood were obtained by tail vein bleeding prior to tumor challenge and every two weeks thereafter. Immediately prior to autopsy 0.7–1.0 ml of blood were obtained by cardiac puncture after animals were anesthetized with sodium pentobarbital. Serum was tested for HBsAg by radioimmunoassay (Aus-RIA II, Abbott Laboratories). Due to the small volumes of blood obtained by venipuncture, 25 or 40 μl of serum were diluted to 200 μl in phosphate-buffered saline, pH 7.5, for the HBsAg assay. Positive results were defined as a $P:N$ ratio > 2.1, where P was the number of gamma counts per minute (cpm) of the test sample and N was the average cpm of 7 negative controls.

Mouse serum was tested for Dane particle DNA polymerase activity by a previously described method [14]. In brief, 40–150 μl of test

serum was centrifuged at 175,000 *g* for 6 hr. The pellet was resuspended in a 75 μl reaction mixture which contained 66 μM dATP, 66 μM dCTP, 66 μM dGTP, 1.3 μM [^{3}H]-dTTP (New England Nuclear, 97 Ci/mmole), 25 mM $MgCl_2$, 66 mM Tris–hydrochloride, pH 7.5, 80 mM NH_4Cl, 0.5% Nonidet P-40 and 0.1% 2-mercaptoethanol. The reaction mixture was incubated at 37°C. At 3 hr, 50 μl of the reaction product were removed and precipitated onto Whatman 3-mm paper disks with 5% trichloroacetic acid. Ten μl were removed from the reaction mixture before [^{3}H]-dTTP was added and served as measurement of background activity. Samples were counted in a Beckman LS-9000 scintillation counter. Plasma from three persons known to contain high levels of Dane particle DNA polymerase activity were included as positive controls. Positive reactions were defined as cpm > 2 times background.

Supernatant of primary cell cultures made from mouse PLC/PRF/5 tumors were also tested for Dane particle DNA polymerase activity. One hundred ml aliquots of supernatant from cells on various days after passage were centrifuged and assayed as described above.

RESULTS

Growth of primary tumor and metastases

Six of 8 (75%) nude mice injected subcutaneously with PLC/PRF/5 cells developed tumor nodules at 13 of 28 (46%) injection sites (Table 1). The mean latency period for visible tumor growth was 28 days (range 18–48 days). These tumors reached large size and in several measured more than 15 mm in diameter. One large, lobular tumor, measuring 28 × 21 × 20 mm, extended from the point of inoculation and encased the left leg such that this extremity could not be moved. The average growth rate for the tumors as a group was 0.35 ± 0.006 mm/day. Primary tumors were removed from two animals and reinoculated subcutaneously into six additional animals. Four of the 6 (67%) developed palpable tumors at 7 of 10 (70%) injection sites in an average of 15 days (range 11–29 days). These tumors grew an average of 0.29 ± 0.012 mm/day.

Tumor developed in 8 of 9 (89%) animals when cells were inoculated into the intraperitoneal cavity (Table 2). Tumor became palpable or visible as opaque nodules beneath the transparent skin over the abdomen in a mean time of 33 days (range 18–72 days) after inoculation. At autopsy the abdominal cavity was characteristically filled with clusters of nodules which grossly distorted the normal anatomy. Loops of bowel were often encased by tumor. Tumor frequently invaded other abdominal organs such as spleen, pancreas, stomach and diaphragm by direct extension.

Microscopically, tumors from subcutaneous or abdominal sites showed pleo-

Table 1. *Subcutaneous inoculation of PLC/PRF/5 cells or tumor into nude mice*

Animal	No. of cells inoculated per site	No. tumors/ No. sites inoculated	Latency period‡ (days)	Maximum GMD§ (mm)	Growth rate‖ (mm/day)	Microscopic pulmonary metastases	Serum HB_sAg (dilution)
S*-1	7.8×10^6	3/4	21, 21, 28	16, 15, 15	NT	—	NT
S-2	7.8×10^6	2/4	21, 28	NT	NT	+	NT
S-3	7.8×10^6	2/4	28, 35	NT	NT	+	NT
S-4	3.2×10^6	1/2	18	23	0.46	NT	4835¶ (1:8)
S-5	3.2×10^6	0/2	—	—	—	—	214 (1:8)
S-6	5.0×10^6	2/4	34, 48	11, 5	0.36, 0.34	—	205 (1:5)
S-7	5.0×10^6	0/4	—	—	—	—	144 (1:8)
S-8	5.0×10^6	3/4	21, 21, 27	8, 11, 35	0.24, 0.35, 0.38	+	12,432 (1:2)
T†-1	—	0/1	—	—	—	NT	179 (1:8)
T-2	—	0/1	—	—	—	NT	208 (1:8)
T-3	—	2/2	13, 29	5, 8	0.18, 0.38	+	232 (1:8)
T-4	—	1/2	13	5	0.16	—	NT
T-5	—	2/2	11, 15	9, 8	0.32, 0.22	—	187 (1:8)
T-6	—	2/2	13, 13	13,10	0.49, 0.32	—	264 (1:8)

*S refers to mice inoculated subcutaneously with PLC/PRF/5 cells.
†T refers to mice given PLC/PRF/5 tumor chips by trocar inoculation.
‡Time until tumor growth was measurable in at least 2 diameters.
§GMD refers to geometric mean diameter.
‖Increase in GMD per day.
¶Positive score is > 396 cpm.

Table 2. Intraperitoneal inoculation of PLC/PRF/5 cells into nude mice

Animal	No. of cells inoculated	Tumor detected at autopsy	Latency period† (days)	Microscopic pulmonary metastases	Day HBsAg first detected in serum	Maximum HBsAg in serum (dilution)
IP*-1	3.2×10^6	—	—	—	—	—
IP-2	3.2×10^6	+	72	+	79	20,158 (1:2)
IP-3	3.2×10^6	+	—	—	58	1735 (1:2)
IP-4	4.1×10^7	+	34	+	34	4494 (1:2)
IP-5	4.1×10^7	+	24	—	24	18,107 (1:5)
IP-6	4.1×10^7	+	24	+	24	4088 (1:5)
IP-7	2.4×10^7	+	28	NT	18	20,011 (1:2)
IP-8	2.4×10^7	+	18	NT	—	176 (1:5)
IP-9	2.4×10^7	+	28	NT	18	13,081 (1:8)

*IP refers to intraperitoneal inoculation.
†Time from inoculation until tumor first visible or palpable.

morphic epithelial cells which were occasionally binucleate, contained numerous mitotic figures and were arranged in a somewhat disorganized but definite cord-like architecture with intervening sinusoidal spaces. In some subcutaneous tumors, bands of lymphocytes invaded the periphery of the tumor. Of particular significance was the fact that 4 of the 9 animals which developed subcutaneous tumors and 3 of the 5 which developed tumor after intraperitoneal inoculation were found to have pulmonary metastases. Tumor cells extended along the lumen of pulmonary veins (Fig. 1). In one animal with pulmonary metastases, tumor cells were present in a subcutaneous lymphatic (Fig. 2) adjacent to a PLC/PRF/5 tumor. Tumor was not found in livers or kidneys. Moreover, livers showed no acute inflammation.

In animals inoculated subcutaneously, tumor cells placed cephalad grew on the average of 0.39 mm/day during linear growth, compared to caudal tumors which grew slower at 0.28 mm/day. This difference was significant ($P < 0.05$, Student's t-test). The average geometric mean diameter (GMD) of cephalad tumors measured 13 mm at 24 days of growth, compared to 8.6 mm for caudal tumors after the same period of measurable growth.

In 4 animals that received PLC/PRF/5 cells subcutaneously and one given an intraperitoneal inoculation, there was no evidence of tumor growth at post-mortem examination, and HBsAg was not detected in serum. Microscopic examination of these animals showed no mouse hepatitis virus infection, which may have augmented the immune response to the tumor cell challenge. Livers and spleens showed no Kupffer cell or lymphoid hyperplasia, respectively. Thus, the reason for the absence of tumor take in these 5 animals was not apparent.

Detection of HBsAg in sera

HBsAg was detected in serum of 2 of 8 mice with tumors produced by subcutaneous injection of PLC/PRF/5 cells or solid tumor (Table 1). One of these 2 animals had the second largest subcutaneous tumor ($21 \times 28 \times 28$ mm), with a GMD of 23 mm, and the other had three tumors, one of which was the largest measured tumor, with a GMD of 35 mm. The antigen was only detected at autopsy and not earlier. The other animals with subcutaneous tumors but without HBsAg in serum all had tumors with GMDs ≤ 13 mm.

Of the 9 tumor-bearing mice which received tumor cells by intra-abdominal inoculation and in whom serum was tested for HBsAg, 8 (89%) had detectable HBsAg (Table 2). These 8 mice had large clusters of peritoneal tumor nodules as well as extensive local organ metastases. The mean $P:N$ in this group was 25.4 at an average serum dilution of 1:4. The one mouse without HBsAg (IP-8) had a small tumor mass with only a few nodules scattered over the peritoneum and no metastases.

To determine whether HBV latent in the PLC/PRF/5 tumors in mice reactivated to replicate *in vivo*, mouse serum was tested for Dane particle DNA polymerase activity. All samples evaluated for HBsAg contained [^{3}H]-labeled acid-precipitable DNA of less than 2 times the background and were therefore considered free of Dane particles. Positive controls gave counts greater than ten times the background.

Primary cultures of subcutaneous tumors

Primary cultures made from subcutaneous tumors showed mixed fibroblast and epithelial

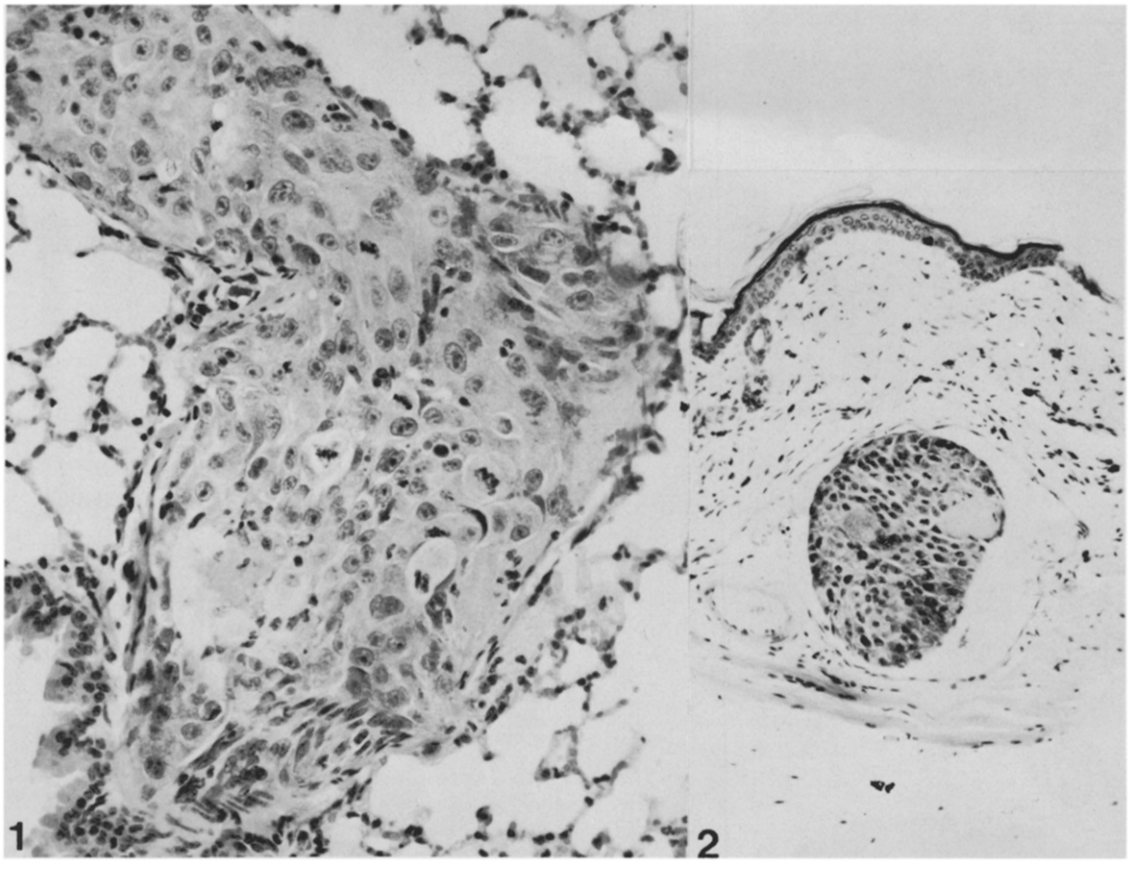

Fig. 1. *PLC/PRF/5 tumor in a pulmonary vascular space of a nude mouse inoculated subcutaneously with tumor cells. H. & E.* × 375.

Fig. 2. *PLC/PRF/5 tumor in a thin walled vessel in subcutaneous tissue at a tumor cell inoculation site. H. & E.* × 192.

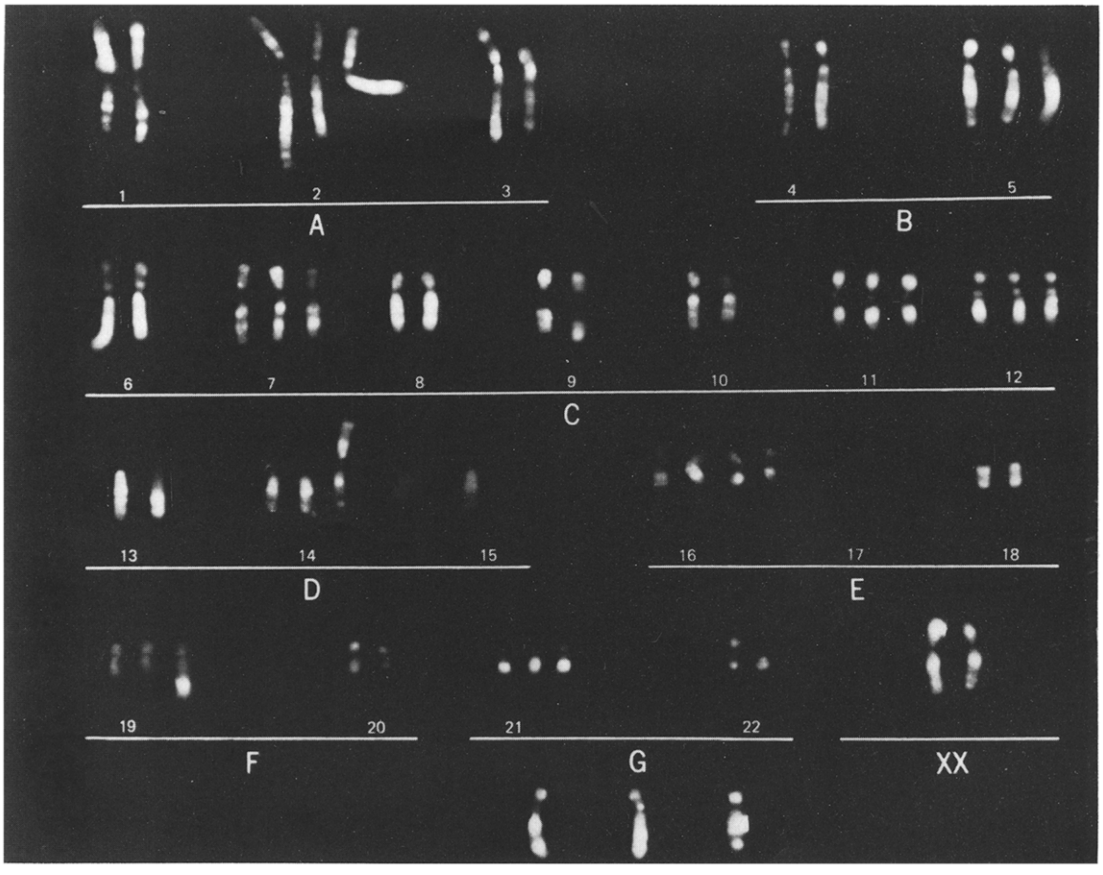

Fig. 4. Q-banded karyotype of a mouse PLC/PRF/5 in its seventh passage in culture.

cells. By the fifth passage, fibroblasts were no longer detected. The epithelial cells showed morphologic and growth characteristics identical to the parent cell line. The supernatant contained high levels of HBsAg but no HBV DNA polymerase activity.

Karyotype

One hundred cells from a mouse PLC/PRF/5 tumor grown in culture were randomly studied in detail. The chromosomes were entirely human, with a modal number of 56 (Fig. 3). The sex chromosomal complement of all cells consisted of two X chromosomes. Several hundred additional cells were scanned for a Y chromosome and none was observed. Numerous autosomal chromosomal abnormalities were also present (Fig. 4). Chromosomes 17 had been lost. At least one apparently normal chromosome was found for each of the other pairs, and for several pairs two or three copies were found. Abnormalities included 2q-, 3p-, 5q-, t(14:14), i(22) and three unidentifiable markers, presumably rearrangements of C-group chromosomes. Chromosomal analysis of PLC/PRF/5 cells from passage number 89 which had not been previously introduced into animals showed the identical karyotype.

DISCUSSION

The results of these experiments indicate that PLC/PRF/5 cells transplanted to athymic nude mice produced tumors in high yield when inoculated either subcutaneously or intraperitoneally. This confirms a preliminary report which indicated that these cells could be successfully grafted to nude mice without application of sublethal irradiation or recourse to surgery for subcapsular implantation of cells beneath the renal capsule [15]. We documented that tumor growth was rapid and that tumor spread locally by direct extension or distantly by vascular dissemination to lung. These features closely resemble characteristics by which primary liver cancer spreads in humans.

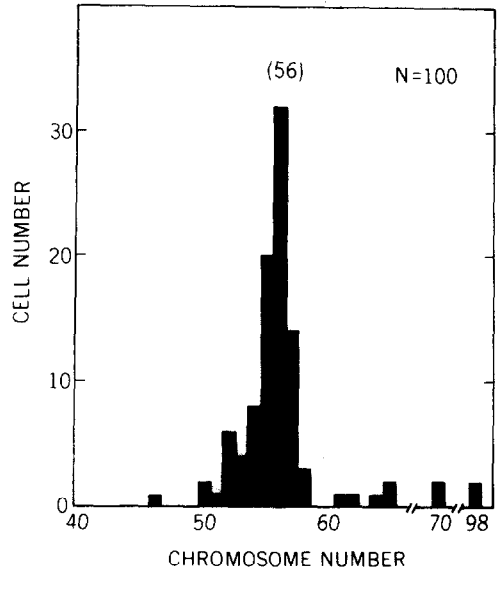

Fig. 3. Histogram of the frequency of chromosome numbers in 100 mouse PLC/PRF/5 tumor cells. The modal chromosome number is 56.

Microscopic examination indicated that these tumors maintained histologic fidelity on serial passage in mice. In addition, morphologic and growth characteristics of primary cell cultures from these tumors remained unchanged after heterotransplantation. Karyotype analysis of tumor cells confirmed that the tumors were of human origin. There was no detectable mouse–human hydridization. However, extensive chromosomal rearrangements and deletions were present. These abnormalities were also present in the parent cell line. The chromosomal pattern has remained remarkably stable over a period of at least 27 passages and did not undergo alteration after passage in the nude mouse. The absence of a Y chromosome probably reflects random chromosomal loss during early passages in which the cell line became established in culture.

At least one cellular protein function, HBsAg production, was maintained *in vivo*. Futhermore, these cells did not revert to an infected state since mouse serum and supernatant of primary cell cultures from PLC/PRF/5 tumors grown in mice contained no Dane particles.

HBsAg was detected in serum of 10 of 17 (59%) tumor-bearing mice and in 89% of those given tumor cells by intraperitoneal injection. The $P:N$ ratio of cpm in the HBsAg radioimmunoassay correlates with antigen concentrations up to 250 μg/ml in human serum [16]. It is therefore possible that HBsAg may have been produced in the other tumor-bearing animals since (1) HBsAg was detected in mice with large tumors and may have been synthesized below detectable levels in those with small tumors, and (2) the small volumes of serum tested required that samples be diluted which may have decreased the sensitivity of the test. Further quantitative assessment will have to be made to determine whether a direct relationship between HBsAg concentration and tumor size truly exists. Such a relationship would provide a convenient means to quantitate tumor growth. This is relevant since the

other major primary liver cancer marker, alpha-fetoprotein, appears to be species specific [17] and would require differentiation of human from mouse alpha-fetoprotein.

The development of pulmonary metastases in 7 of 14 (50%) tumor-bearing nude mice inoculated with PLC/PRF/5 is a unique feature of these experiments. Distant metastasis of human tumor transplanted to nude mice is rare [18]. Moreover, pulmonary spread has not heretofore been reported in the limited experience with PLC/PRF/5 tumors produced in nude mice. In humans with primary liver cancer distant metastases are detected primarily in lung and are present in 30–50% of patients [19]. Tumor reaches the lung by hematogenous dissemination of microemboli or direct extension of tumor along the inferior *vena cava*. In our experiments the presence of tumor cells in pulmonary veins confirms that vascular dissemination and embolization occurs in nude mice as well. However, we were surprised that lung metastases occurred when PLC/PRF/5 cells were inoculated subcutaneously. The fact that tumor was present in a subcutaneous lymphatic adjacent to an inoculation site indicates that vascular dissemination to lung may occur in some instances by microinvasion of tumor cells first into lymph. Lymph node metastases were not present, but tumor cells may have entered the peripheral blood via the thoracic duct. Regardless, the presence or absence of pulmonary metastases provides a valuable marker of the extent of disease. This should facilitate evaluation of the effectiveness of new chemotherapeutic agents in controlling the spread of tumor *in vivo* before such agents are empirically tested in humans.

Our experiments indicate that regional differences in tumor growth occur when cells are inoculated in the anterior, lateral thoracic region compared to the posterior, lateral lumbar region. These findings support a previous study that showed tumor cells from monolayer cultures grow more rapidly when injected in the cephalad areas of nude mice [20]. Therefore, care will have to be taken to consider geographical differences of injection sites in determination of growth kinetics or efficacy of drug therapy in PLC/PRF/5 and possibly other human primary liver cancers transplanted to nude mice.

Bassendine *et al.* [11] successfully transplanted PLC/PRF/5 cells subcutaneously into nude mice irradiated with sublethal doses of cobalt 60. Only one of 19 normal animals given $\leq 5 \times 10^6$ cells and 3 of 8 given 10^7 cells per injection site developed tumors. The greater take rate of non-irradiated mice in our study may have been due to several differences in experimental design. First, we used similar cell inocula, but our concentrations were based on the number of viable cells. In our study 10–15% of harvested cells were stained by trypan blue and thus were non-viable. If the investigators included these non-viable cells in their inocula size, they may have injected fewer viable cells than we did. Second, different strains of nude mice may be more or less susceptible to the same test tumor. In addition, mouse hepatitis virus infection often present in nude mice stocks inhibits growth of some tumors [21, 22]. Third, we intentionally inoculated only mice less than 6 weeks of age since natural killer cell cytotoxicity is increased in older animals [23, 24]. It is possible that if the investigators used 'older' animals this could explain the significant difference in tumor susceptibility in their non-irradiated nude mice and ours.

The nude mouse should prove useful in the study of human primary liver cancer. The high tumor take rate of 78% in our experiments (tumor developed in 18 of 23 animals tested) suggests that other hepatitis B-related primary hepatocellular cancer cell lines may be produced by direct inoculation of fresh tumor into nude mice. Production of additional cell lines should facilitate investigation of the causal relationship of HBV to this neoplasm. In particular, it will be important to determine if the incomplete genomic expression of HBV in PLC/PRF/5 and Hep 3B cells is characteristic of the transformed state of malignant hepatocytes in persons with HBV infection. In addition, the absence of thymus-dependent lymphocytes in nude mice should provide a suitable model to investigate *in vivo* the tumor immunology of human liver cancer. Finally, the growth characteristics of PLC/PRF/5 tumors and presence of serum HBsAg in nude mice closely mimic features of primary liver cancer in humans. Hence, the nude mouse should become a valuable model to test new forms of therapy for human hepatocellular cancer.

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REFERENCES

1. KEW MC. Hepatoma and the HBV. In: VYAS GN, COHEN SN, SCHMID R, eds. *Viral Hepatitis*. Philadelphia, Franklin Institute Press, 1978, 439–450.

2. ILARDI CF, YING YY, ACKERMAN LV, ELIAS JM. Hepatitis B surface antigen and hepatocellular carcinoma in The People's Republic of China. *Cancer* 1980, **46**, 1612–1616.
3. KEW MC, RAY MB, DESMET VJ, DESMYTER J. Hepatitis-B surface antigen in tumour tissue and non-tumorous liver in black patients with hepatocellular carcinoma. *Br J Cancer* 1980, **41**, 399–406.
4. ALEXANDER J, BEY E, WHITCUTT JM, GEAR JHS. Adaptation of cells derived from human malignant tumours to growth *in vivo*. *S Afr J Med Sci* 1976, **41**, 89–98.
5. ADEN DP, FOGEL A, PLOTKIN S, DAMJANOV I, KNOWLES BB. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature (Lond)* 1979, **282**, 615–616.
6. MACNAB GM, ALEXANDER JJ, LECTASAS G, BEY EM, URBANOWICZ JM. Hepatitis B surface antigen produced by a human hepatoma cell line. *Br J Cancer* 1976, **34**, 509–515.
7. MARION PL, SALAZAR FH, ALEXANDER JJ, ROBINSON WS. State of hepatitis B viral DNA in a human hepatoma cell line. *J Virol* 1980, **33**, 795–806.
8. GERBER MA, GARFINKEL E, HIRSCHMAN SZ, THUNG SN, PANAGIOTATOS T. Immune and enzyme histochemical studies of a human hepatocellular carcinoma cell line producing hepatitis B surface antigen. *J Immunol* 1981, **126**, 1085–1089.
9. TWIST EM, CLARK HF, ADEN DP, KNOWLES BB, PLOTKIN SA. Integration pattern of hepatitis B virus DNA sequences in human hepatoma cell lines. *J Virol* 1981, **37**, 239–243.
10. KNOWLES BB, HOWE CC, ADEN DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* 1980, **290**, 497–499.
11. BASSENDINE MF, ARBORGH BAM, SHIPTON U *et al.* Hepatitis B surface antigen and alpha-fetoprotein secreting human primary liver cell cancer in athymic mice. *Gastroenterology* 1980, **79**, 528–532.
12. CASPERSSON T, LOMAKKA G, ZECH L. 24 fluorescence patterns of human metaphase chromosomes—distinguishing characters and variability. *Hereditas* 1971, **67**, 89–102.
13. SEABRIGHT M. A rapid technique for human chromosomes. *Lancet* 1971, **ii**, 971–972.
14. SATTLER FR, ROBINSON WS. Hepatitis B viral DNA molecules have cohesive ends. *J Virol* 1979, **32**, 226–233.
15. DESMYTER J, RAY MB, BRADBURNE AF, ALEXANDER JJ. Human HBsAg-positive hepatoma in nude athymic mice. In: VYAS GN, COHEN SN, SCHMID R, eds. *Viral Hepatitis*. Philadelphia, Franklin Institute Press, 1978, 459–460.
16. LING CM, DECKER RH, FEOMMEL RS, OVERBY LR. Comparison of confirmation methods of hepatitis B antigen and the nature of false positives detected by ^{125}I immunoglobulins. *J Lab Clin Med* 1975, **86**, 690–699.
17. RUOSLAHTI E, SEPPÄLÄ M. α-Fetoprotein in cancer and fetal development. *Adv Cancer Res* 1979, **29**, 275–346.
18. GIOVANELLA BC, STEHLIN JS, WILLIAMS LJ, JR, LEE S-S, SHEPARD RC. Heterotransplantation of human cancers into nude mice. *Cancer* 1978, **42**, 2269–2281.
19. MORTEL CG. The liver. In: HOLLAND JF, FREI E, III, eds. *Cancer Medicine*. Philadelphia, Lea and Febiger, 1973, 1541–1547.
20. KYRIAZIS AA, KYRIAZIS AP. Preferential sites of growth of human tumors in nude mice following subcutaneous transplantation. *Cancer Res* 1980, **40**, 4509–4511.
21. KYRIAZIS AP, DIPERSIO L, MICHAEL JG, PESCE AJ. Influence of the mouse hepatitis virus (MHV) infection on the growth of human tumors in the athymic mouse. *Int J Cancer* 1979, **23**, 402–409.
22. TAMURA T, SAKAGUCHI A, KAI C, FUJIWARA K. Enhanced phagocytic activity of macrophages in mouse hepatitis virus-infected nude mice. *Microbiol Immunol* 1980, **24**, 243–247.
23. HERBERMAN RB, NUNN ME, LARVIN DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *Int J Cancer* 1975, **19**, 216–219.
24. HERBERMAN RB. Natural cell-mediated cytotoxicity in nude mice. In: FOGH J, GIOVANELLA BC, eds. *The Nude Mouse in Experimental and Clinical Research*. New York, Academic Press, 1978, 136–166.