Virchows Archiv A Pathol Anat Histopathol (1988) 412:595–606

Virchows Archiv A
Pathological Anatomy and Histopathology

Comparative morphology and tumourigenicity of human hepatocellular carcinoma cell lines in athymic rats and mice

Daniel Shouval [1], Lucia Schuger [2], Itzhak S. Levij [2], Lola M. Reid [3], Zvi Neeman [2], and David A. Shafritz [3]

[1] Liver Unit, Department of Medicine A, [2] Department of Pathology, Hadassah University Hospital, Jerusalem 91120, Israel
[3] The Marion Bessin Liver Research Center and Departments of Medicine, Cell Biology and Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461, USA

Summary. Four human hepatoma cell lines PLC/PRF/5, Hep G2, Sk-Hep 1 and Mahlavu were inoculated subcutaneously into athymic Balb/c nude mice and N/NIH outbred nude rats, producing well encapsulated tumours. The 4 hepatoma tumour types in the athymic rodents differ morphologically. PLC/PRF/5 and Hep G2 cells are well differentiated polygonal cells which resemble normal hepatocytes. Tumour arrangement is characterized by solid masses and trabeculae while stromal support is minimal. In contrast, Mahlavu and Sk-Hep 1 tumours have a sarcomatous appearance and consist of spindle shaped cells arranged in solid masses with a rich stromal support. Tumourigenicity of hepatoma cells in the athymic rodents was dependent on injected cell type, inoculation density, relative immunocompetence of the host and the species of animals used. In nude mice, Sk-Hep 1 cells were the most tumourigenic, while Hep G2 cells were tumourigenic only at very high inoculation densities. In nude rats, which were more resistant to tumour formation, PLC/PRF/5 cells were the most tumourigenic. Pre-treatment of athymic mice and rats with total body irradiation resulted in enhanced tumourigenicity for all hepatoma cell lines tested. This was manifested as increased "take" rates, a decreased latency from tumour cell injection to tumour detection, increased tumour weight, and for PLC/PRF/5 cells an increased invasiveness to adjacent body cavities. Furthermore, following irradiation, the minimal number of injected cells required to produce subcutaneous tumours was markedly reduced in both animal species, regardless of tumour cell type. The protocols described enable the reproduceable growth of human hepatoma tumours in athymic rodents.

Key words: Hepatoma – Athymic mice – Athymic rats

Offprint requests to: D. Shouval

Introduction

Several human hepatocellular carcinoma (HCC) cell lines have been established in culture (Aden et al. 1979; Chang 1954; Chen 1964; Das et al. 1980; Doi et al. 1975; Fogh et al. 1977a; Macnab et al. 1976; Nakabayashi et al. 1984; Owens et al. 1976; Prozesky et al. 1972; Watanabi et al. 1983) and represent a spectrum of transformed cells of hepatic origin with varying grades of differentiation. Growth of such HCC cells as subcutaneous (s.c.) solid tumours in athymic mice has been only partially successful due to relatively low tumourigenicity of hepatoma cells in these animals (Hirohashi et al. 1977; Hirohashi et al. 1979; Knowles and Aden 1980; Shouval et al. 1981; Shouval et al. 1983). Experimental model systems utilizing HCC tumours in athymic mice have provided a tool for the study of HCC, especially with regard to its association with persistent hepatitis B virus infection (Shouval et al. 1981; Shouval et al. 1983). The purpose of the present communication is to characterize and compare the morphology and tumourigenicity of 3, as yet unreported, human HCC cell lines in athymic rats and mice and to compare the data with those obtained in the previously described human hepatoma cell line PLC/PRF/5 (Shouval et al. 1983).

Materials and methods

A colony of athymic nude rats (N:NIH strain) was established from a breeding nucleus obtained from Dr. Carl T. Hansen, the Veterinary Resources Branch, Division of Research Services, National Institute of Health, Bethesda, MD. Homozygous NIH rnu/rnu males were bred with heterozygous NIH rnu/+ females as described for athymic mice (Shouval 1981). Animals were kept in isolation within barrier quarters, under positive air pressure, with HEPA filtered air. Rats were housed in cages with individual filter bonnets for a period of up to 2 years. Twenty-eight to 50 day old animals were used for tumour transplantation.

A breeding colony of athymic Balb/c mice was obtained from Bomholtgaard Ltd., Denmark. Mice were bred and maintained as described (Shouval 1981). Four to six week old mice were used for experiments.

Human hepatoma cell lines PLC/PRF/5 (Alexander et al. 1978; Sk-Hep 1 (Fogh et al. 1977a) and Mahlavu (Prozesky et al. 1972) were grown in culture using RPMI 1640 supplemented with L-Glutamine, non essential amino acids, penicillin, streptomycin, Fungizone® and 10% fetal bovine serum (FBS), referred to as complete medium as previously described (Shouval et al. 1981). The 3 hepatoma cell lines were at passage 100–200, have a human karyotype and were originally double cloned in our laboratories. Hep G2 cells at passage 25–55 in our hands, obtained from Dr. B. Knowles (Wistar Inst., Philadelphia, PA, USA) were grown in Delbeco's Modified Eagles medium containing 10% FBS and supplements as described for RPMI 1640 medium (Shouval et al. 1981).

Subconfluent cultures were treated by trypsin-EDTA and suspended in complete medium (Shouval et al. 1981). Viable cells, as determined by trypan blue exclusion, were injected s.c. at the desired concentration in 0.2 ml serum free medium into the flank of athymic rats and mice as described (Shouval et al. 1981).

Athymic mice and rats were irradiated with 400 and 600 rad respectively (Orthovoltage X-ray source, 175 KVP, HVL 0.2 mm copper, Philips FRG), 10 days before tumour cell injection (Shouval et al. 1983).

Animals were examined every 48 h for tumour appearance. At the end of experiments, they were exsanguinated under tribromoethanol anesthesia (Shouval et al. 1981) and weighed. Tumour, liver, spleen, lungs, kidneys and lymph nodes were excised and weighed. Organ and tumour weights were expressed in mg/g body weight. Tissues were fixed in 10% buffered formaldehyde and embedded in paraffin. Sections (5 micron) were stained with haematoxylin eosin. Tumour sections were also stained for reticulin (Gomori), connective tissue (Masson trichrome), glycogen (PAS, PAS Diastase), iron (Prussian blue), mucin (Mucicarmin), fat (oil red), bile (Fouchet), copper (rubeanic acid), HBsAg (anti-HBs) and alpha fetoprotein (anti-AFP). All specimens were examined by 3 independent observers.

For electron microscopy tumour tissue was fixed in 4% formaldehyde – 1% glutaraldehyde solution (McDowell 1978). Samples were rinsed in 0.1 M cocodylate buffer containing 8.5% sucrose and post fixed in 2% OsO_4 in veronal acetate buffer, pH 7.4. En block staining with uranyl acetate was then carried out followed by dehydration through graded ethanol solutions and embedding in Epon 812. Thin sections were cut with an LKB Ultratome III, mounted on bare grids and stained with lead citrate. Specimens were examined and photographed at 80 KV using a Jeol 1000 CX electron microscope.

Blood was collected from mice by retrobulbar puncture and from rats through the ventral tail vein. It was allowed to clot over night at 4° C. HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc and alpha fetoprotein (AFP) were measured in serum by RIA (Abbott Laboratories, N. Chicago, Ill. and Serrono, Italy) (Shouval et al. 1981).

Results

Subcutaneous injection of hepatoma cells of the various types into athymic mice and rats produced well encapsulated tumours with a similar morphological appearance of the same tumour in both animal strains. Only tumour sections from athymic mice were examined, since tumours from athymic rats usually showed extended necrosis.

With PLC/PRF/5 cells the tumour was predominantly of trabecular patterns and resembled normal liver parenchyma (Fig. 1A). Most of the trabeculae consisted of 2 or 3 cell layers. Solid areas were also observed. The component cells were polygonal or cuboidal with vesicular nuclei containing one or two eosinophilic nucleoli. There was much pleomorphism and a few cells were multinucleated. Mitoses were numerous and often atypical. A characteristic histological feature was the presence of spaces filled with blood and debris. These spaces had no endothelial lining and resembled the findings in peliosis hepatis (Fig. 1B). These findings were further corroborated by the macroscopic recognition of such foci at autopsy. Between the trabeculae there was a delicate network of sinusoids with very few reticulin fibers (Fig. 1C). Necrotic areas, randomly distributed, were common and apoptotic bodies were also occasionally found. Fine droplets of fat were present in many of the tumour cells, especially around the necrotic areas. Inflammatory infiltration of the fibrotic capsule was almost absent. Ultrastructural analysis of PLC/PRF/5 tumours in nude mice revealed cells with numerous short microvilli filling bile canaliculi. Junctional complexes were seen between the lumen forming cells (Fig. 1D).

With Hep G2 cells the tumour presented a uniform, solid pattern. The component cells were pleomorphic but resembled hepatocytes (Fig. 2A). Most cells showed a large, hyperchromatic nucleus while in the remainder the nucleus was vesicular with a prominent nucleolus. A few cells were multinucleated. Mitoses were numerous and often atypical. Scattered cells contained neutral mucus. Random areas of necrosis as well as apoptotic bodies were observed. Intracellular fat was common near necrotic areas. The stromal support consisted of strikingly few fine septa composed of fibers which stained for collagen and reticulin (Fig. 2C). There were also areas reminiscent of peliosis as seen in PLC/PRF/5 tumours (Fig. 2B). Foci of in-

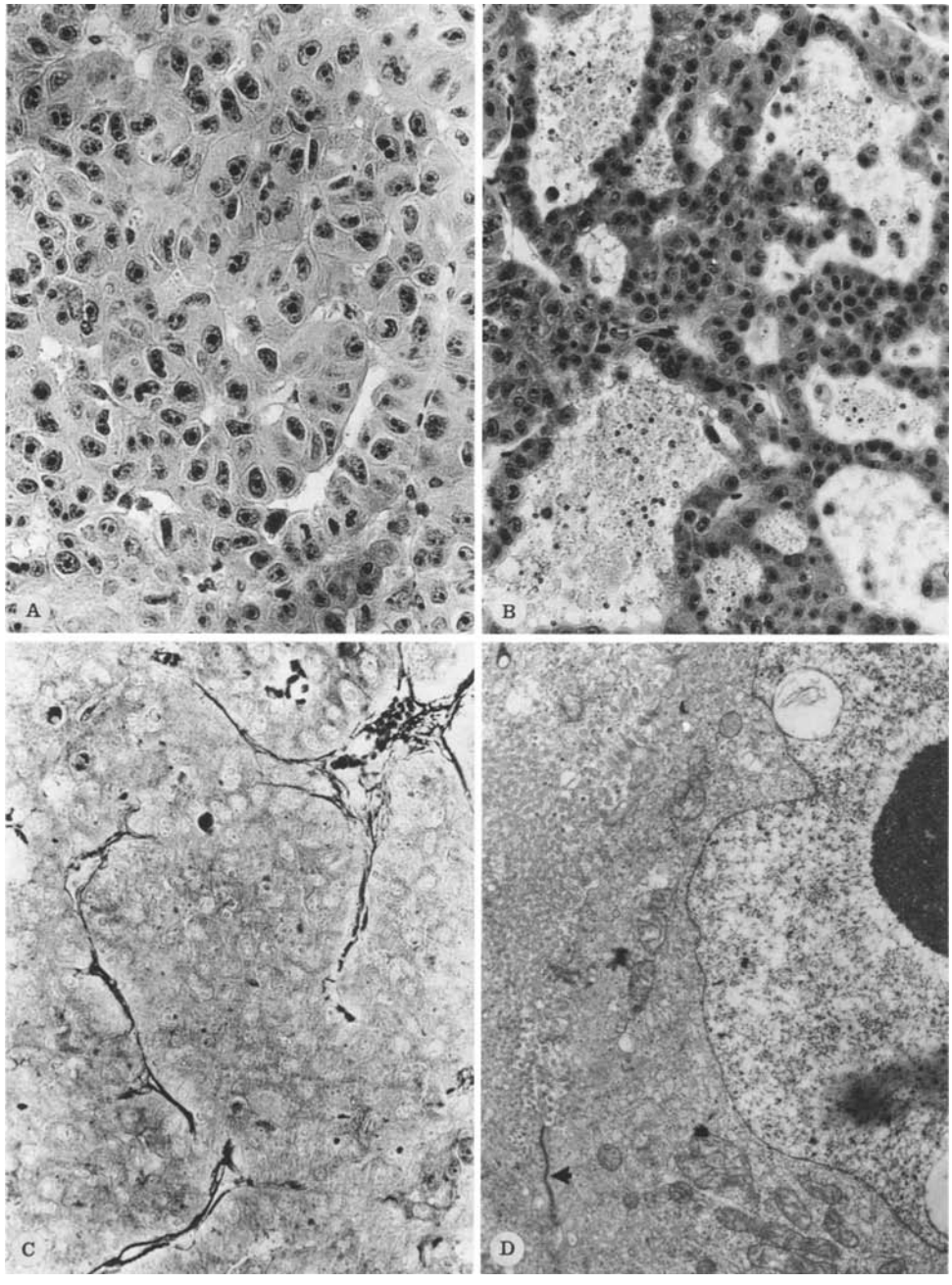

Fig. 1. PLC/PRF/5. **A** Well differentiated tumour cells with a trabecular pattern. Trabeculae are separated by compressed sinusoids lined by endothelium (H&E ×280); **B** Tumour cell trabeculae surrounding spaces filled with blood and cell debris. Spaces are devoid of endothelial lining with a resemblance to peliosis hepatis (H&E ×112); **C** The tumour is poor in supportive stroma consisting of a small number of fibers staining for reticulin (Gomori ×216); **D** Electron micrograph showing a cell junction (*arrowhead*) and a bile duct lumen resembling an acinus (×4816)

flammatory mononuclear cells were present at the margins. Electron-microscopically there were intercellular acinar lumens formed by cells coupled by junctional complexes (Fig. 2D).

The Sk-Hep 1 cell tumours consisted of spindle shaped cells (Fig. 3A) and showed a sarcomatous appearance (Fig. 3B), with an occasional nodular pattern (Fig. 3C1). The nuclei were ovoid or elongated. There were generally 2 or more nucleoli per nucleus, and mitoses were abundant. The stroma contained a dense network of fibers staining for reticulin (Fig. 3C2). Necrosis was present in the centre of the tumour, surrounded by fat containing cells. An inflammatory infiltrate composed of

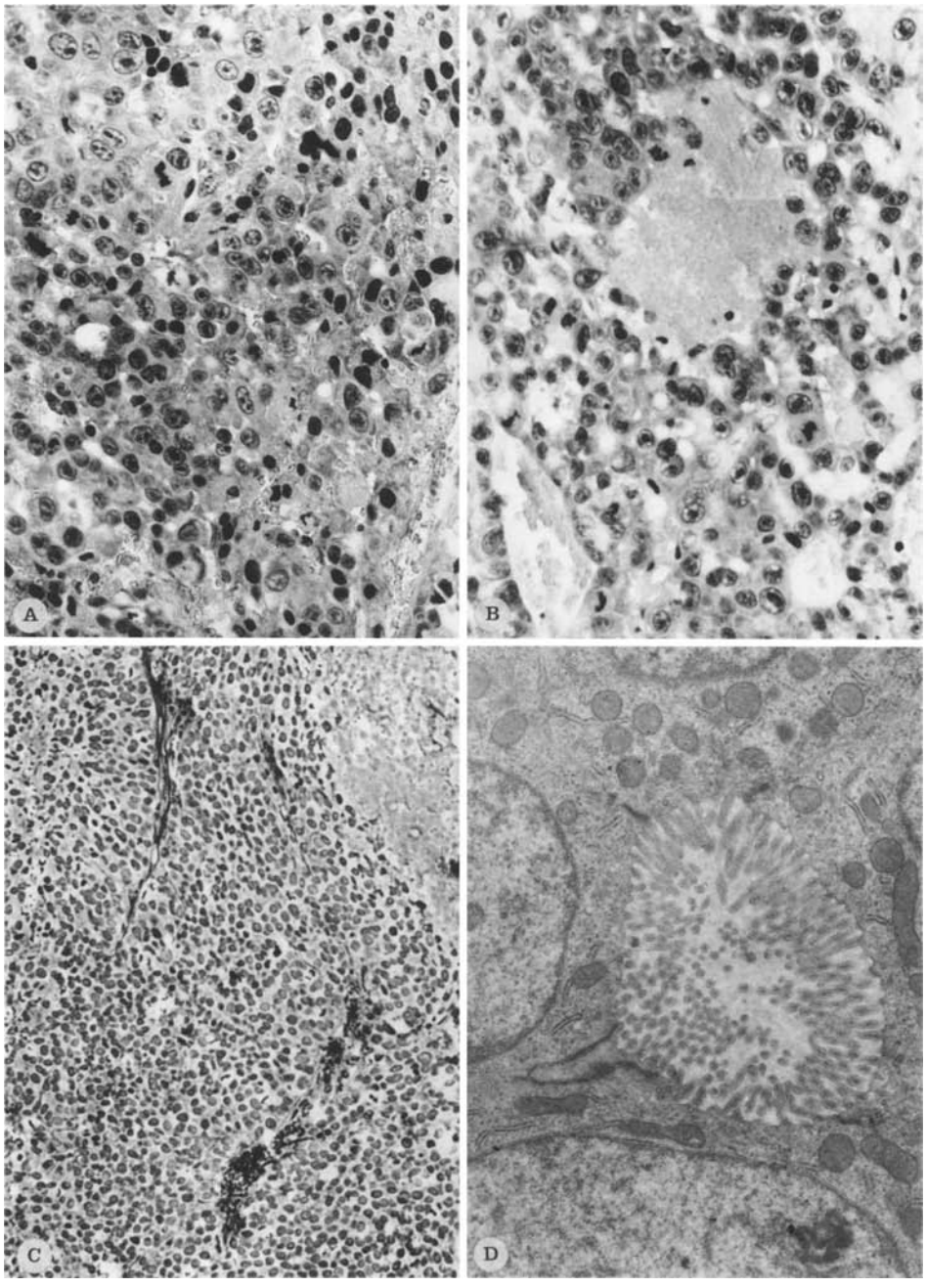

Fig. 2. Hep G2. **A** Moderately differentiated HCC cells growing in a solid pattern (H&E ×280); **B** A space filled with blood and debris as shown in Fig. 1B (H&E ×280); **C** Cellular area of the tumour with only minimal stromal support (Gomori ×96); **D** Electron micrograph showing a tumour cell acinus with a number of intercellular junctions (×8480)

mononuclear cells and neutrophils was observed at the margins. Electron-microscopic examination regularly revealed cell junctions as well as bile canaliculi (Fig. 3D).

Tumours composed of Mahlavu cells also had a sarcomatous appearance (Fig. 4A). The cells were elongated and arranged in solid masses or bands. The nuclei were large, ovoid or elongated, uniform in size and contained one or more prominent nucleoli. The cytoplasm was pale and scanty; some apoptotic bodies were present and mitoses were numerous. The supportive stroma was abundant, rich in fibroblasts and fibers staining for connective tissue and reticulin (Fig. 4A–C). Sinusoids were scanty and hardly recognizable. Necrosis, when present, occurred only in the central area of the tumour. A few cells contained large cytoplasmic fat vacuoles. A few mononuclear inflam-

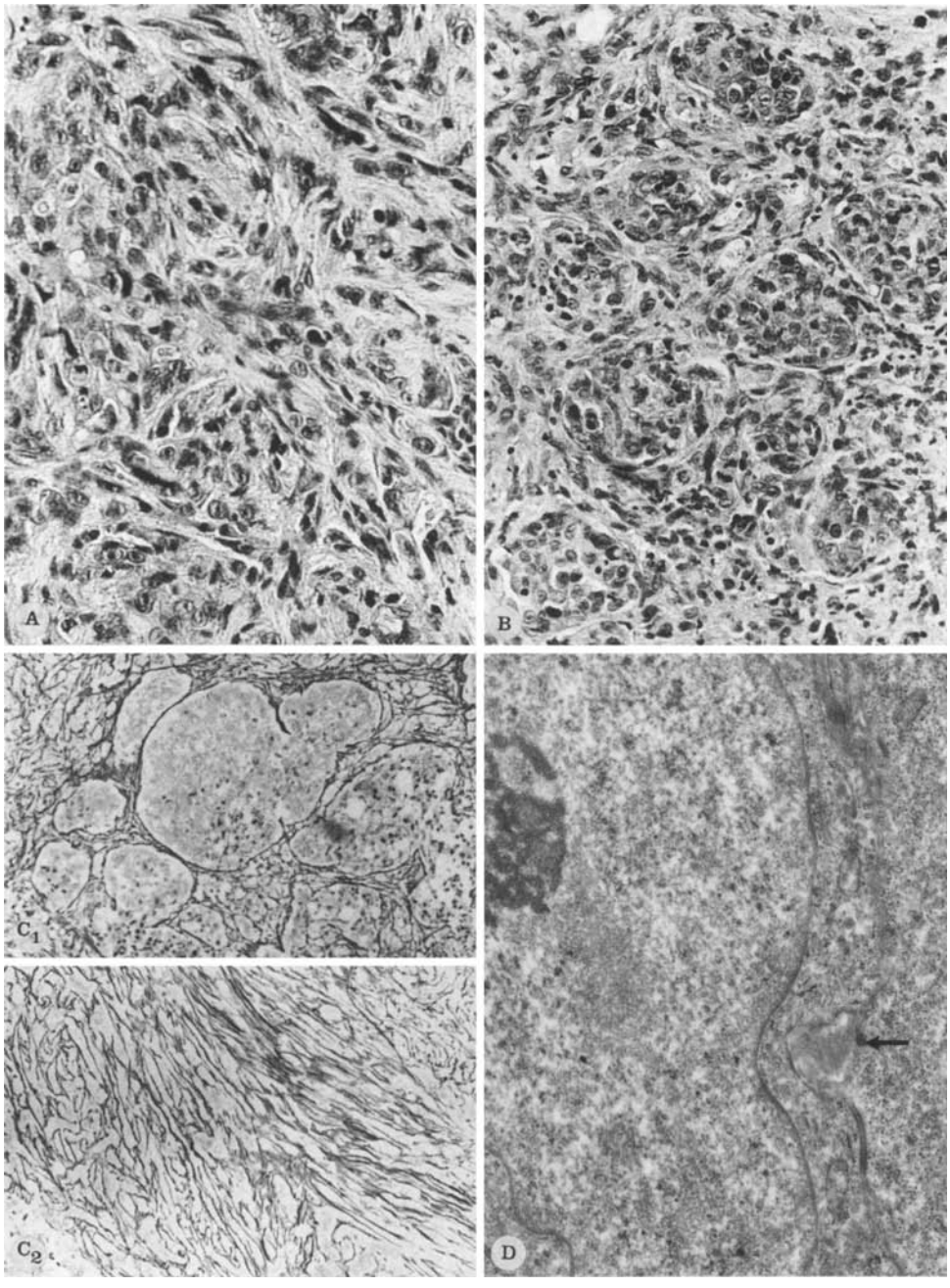

Fig. 3. *Sk-Hep 1.* **A** Poorly differentiated tumour with a sarcomatous pattern consisting of spindle cells organized in whorls (H&E ×280); **B** The tumour shows areas of various degrees of differentiation where the cells are arranged in a nodular pattern (H&E ×216); **C1** Fiber network surrounding tumour nodules (Gomori ×112); **C2** In this part of the tumour the fibers form a dense network in a sarcomatous pattern (Gomori ×112); **D** Electron micrograph showing part of tumour cells adjacent to a bile canaliculus (*arrow*) (×13120)

matory cells were present at the margins. Ultrastructurally, the cells contained large oval nuclei. In the cytoplasm, prominent rough endoplasmic reticulum and aggregates of glycogen particles were seen. In this tumour no acini, lumens or bile canaliculi were present, although neighbouring tumour cells were interlocked by desmosomes and interdigitations of the plasma membranes (Fig. 4D). A comparative tabulation of the light microscopic characteristics of the 4 tumour types is presented in Tables 1 and 2.

Subcutaneous injection of hepatoma cells into athymic rodents led to the growth of localized, well encapsulated tumours in untreated animals. Tu-

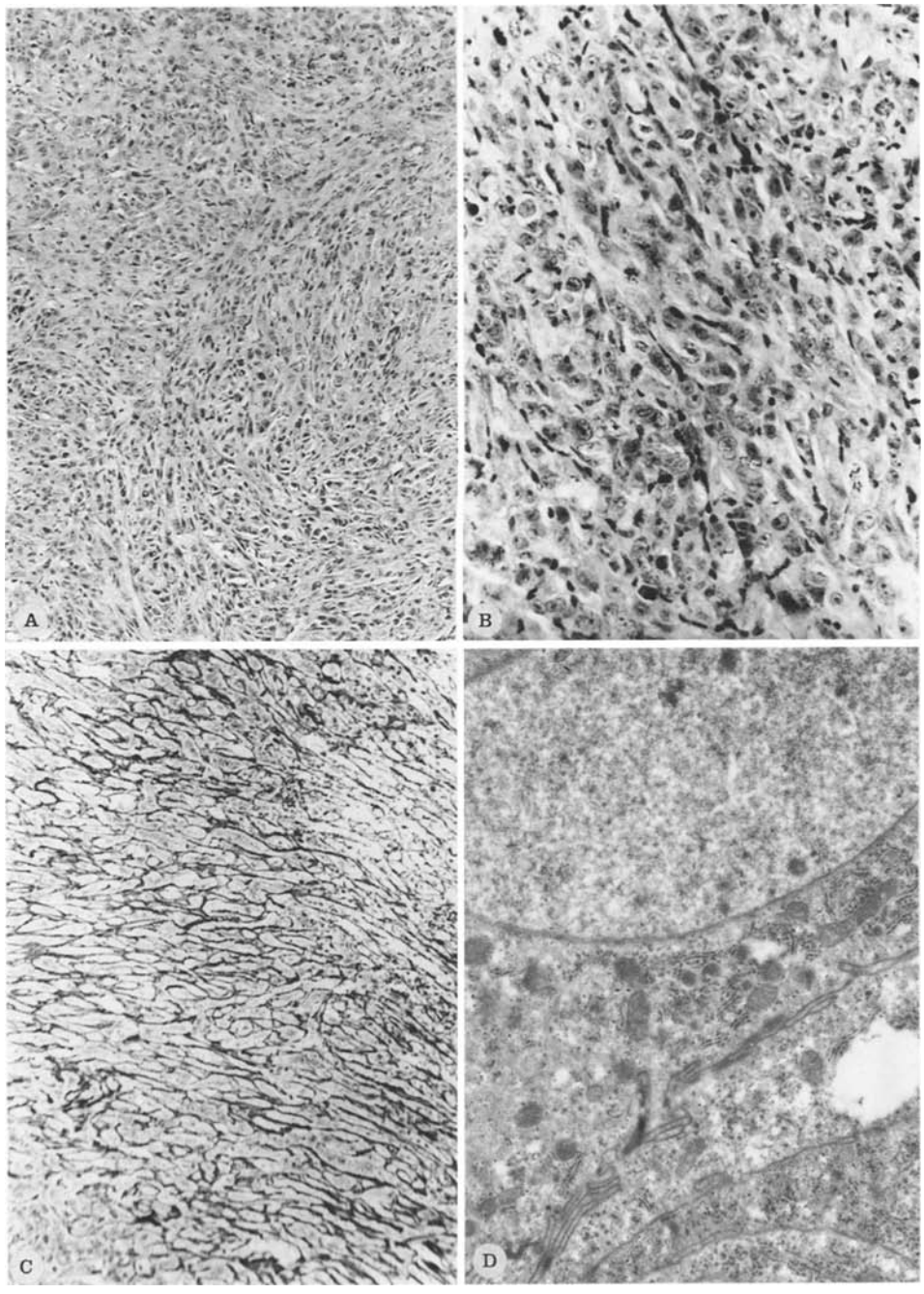

Fig. 4. Mahlavu. **A** Poorly differentiated HCC of spindle cell type with a sarcomatous pattern (H&E ×72); **B** Despite the poor differentiation of tumour cells, their hepatocellular origin is still recognizable in the appearance of nuclei (H&E ×216); **C** An excessively rich network of fibers extends between tumour cells and a trabecular pattern becomes apparent (Gomori ×112); **D** Electron micrograph showing adjacent parts of two tumour cells. Note the interdigitations between the adjoining cell plasma membrane (×7000)

mourigenicity was however dependent on tumour cell type, number of injected cells and immunocompetence of the host. Furthermore, hepatoma cells were more tumourigenic in athymic mice when compared with athymic rats as determined by the number of injected cells required to produce tumours and by latency from cell injection to tumour detection (Tables 3, 4). Irradiation of rats or mice 10 days prior to tumour cell injection, caused significant augmentation of tumourigenicity of hepatoma cells. This was manifested by a decreased cell inoculation density required to pro-

Table 1. Light microscopic characterization of hepatoma cell tumours in athymic rodents

	PLC/PRF/5	Hep G 2	Sk-Hep 1	Mahlavu
Tumour cell arrangement	Solid masses; trabeculae	Solid masses	Solid masses forming whorls and nodules	Solid masses
Cell shape	Polygonal monomorphic	Polygonal pleomorphic	Spindle pleomorphic	Spindle monomorphic
Fibrous stroma	Negligible	Fine septa	Coarse and fine fibers with pericellular distribution	Fine fibers with pericellular distribution
Reticulin network	Scanty	Scanty	Dense network	Dense network
Inflammatory infiltrate	Negligible	Moderate mononuclear cells	Moderate mononuclear cells, neutrophils	Scanty; mononuclear cells
Blood filled spaces	Present	Present	Absent	Absent

Table 2. Staining properties of hepatoma tumours in athymic rodents

	PLC/PRF/5	Hep G 2	Sk-Hep 1	Mahlavu
Masson Trichrome	Minimal stroma	Minimal stroma	Broad bands producing a nodular pattern	Delicate network surrounding individual cells and groups of cells
Gomori	Scarce reticluin fibers	Scarce reticulin fibers	Diffuse network surrounding single cells and groups of cells	Same as Sk-Hep al
PAS	No PAS positive material	Scattered cells intracytoplasmic granules	Intracytoplasmic granules adjacent to necrotic areas	Foci of intra-cytoplasmic granules
Mucicarmin	Negative	Scattered cells with intracytoplasmic vacuoles	Negative	Negative
Oil red	Diffuse small fat vacuoles	Small and large fat vacuoles	As in Hep G 2	Few large fat vacuoles
Perl's stain	Negative for iron	Negative for iron	Negative for iron	Positive for iron in scattered macrophages
PAP technique for HBsAg	Positive	Negative	Negative	Negative
PAP technique for Alpha feto-protein	Positive	Positive	Negative	Negative

Staining for bile (Fouchet), copper (rubeanic acid), glycogen (*PAS*) and *PAS*-Diastase were all negative for the 4 tumour types

duce tumours, a decreased latency, increased "take" rates and by the increased weight of tumours in irradiated animals (Tables 3, 4). In addition, irradiation caused an increased local invasiveness of tumours, manifested by penetration of neoplasms through the fibrotic capsules and into various adjacent body cavities. This phenomenon was most pronounced in irradiated mice injected with PLC/PRF/5 cells.

In general PLC/PRF/5 cells were the most tumourigenic in untreated athymic rats, followed by Sk-Hep 1 cells. In contrast, tumours were only rarely generated by s.c. injection of Mahlavu cells, and then usually regressed. Since Hep G2 cells were extremely difficult to grow in athymic mice (Table 4), these experiments were not performed in athymic rats. Irradiation of athymic rats caused increased "take" rates of all 3 hepatoma cell lines. In nude rats, PLC/PRF/5 cells were the most tumourigenic while Mahlavu tumours were significantly smaller and slower to grow (Table 3). In athymic mice, Sk-Hep 1 cells were the most tumourigenic, followed by PLC/PRF/5, Mahlavu and Hep G2 cells.

Metastases were rarely observed following s.c. hepatoma cell injection, regardless of tumour cell type used and irrespective of irradiation or animal strain. Even intravenous injection of $2–5 \times 10^6$

Table 3. Tumourigenicity of human hepatoma cells in nude rats

Hepatoma cell line injected	No. inj. cells $\times 10^6$	Treatment[a]	Latency[b] (days)	Tumour frequency: No. of rats with neoplasm / No. of rats injected	Mean tumour weight (g)
PLC/PRF/5	1–4	None	–	0/14	–
	5	None	24–31	1/5	–
	7–10	None	14–29	14/18	7.5
	20	None	16–20	2/3	–
	1–4	X-irrad	15–27	6/9	–
	5	X-irrad	15–23	4/4	–
	7–10	X-irrad	12–25	10/10	11.5
	20	X-irrad	15	2/2	–
Sk-Hep 1	1–4	None	–	0/8	–
	5	None	63	1/6[c]	–
	7–10	None	33–36	1/14	–
	20	None	31–34	1/3	6.4
	1–4	X-irrad	36–48	3/10	–
	5	X-irrad	32–50	4/8	–
	7–10	X-irrad	14–24	8/10	18.7
Mahlavu	1–4	None	–	0/7	–
	5	None	–	0/6	–
	7–10	None	50	1/10[c]	0.2
	1–3	X-irrad	–	0/6	–
	4	X-irrad	28–38	3/4	–
	5–10	X-irrad	24–32	6/6	0.5
Hep G2	N.D.	–	–	–	–

[a] *X-irrad:* Rats were irradiated with 600 rad 10 days prior to tumour cell injection
[b] *Latency* from s.c. tumour cell injection to tumour detection
[c] Tumour regressed
N.D. = Not Done
Animals were sacrificed 2–3 weeks after tumour detection

PLC/PRF/5 and of Sk-Hep 1 cells produced pulmonary metastases in only 3/10 and 1/10 injected animals respectively. Intraperitoneal injection of 10^7 PLC/PRF/5 or Sk-Hep 1 cells caused intra-abdominal tumour growth in both mice and rats that were however, completely resistant to i.p. injection of Mahlavu and Hep G2 cells.

Tumours in treated animals (mice or rats) injected with 10^7 cells s.c., regardless of cell type, continued to grow for a period of 30–50 days and then stabilized in size. No deaths were observed among tumour bearing athymic rats within 3 months after tumour detection. Death was also rare among the athymic mice. During that period animals lost up to 30% of body weight, weight loss which was proportional to tumour size. Irradiation was generally well tolerated with development of occasional petechiae in up to 10% of animals, but with no significant mortality. At autopsy, PLC/PRF/5 tumour bearing rats showed hepatomegaly with mean liver weight of 60 ± 15 mg/g body weight ($n=5$ $p<0.05$) when compared with 34 ± 7 mg/g in control rats ($n=5$). Massive splenomegaly was observed in Sk-Hep 1 and Mahlavu injected non-irradiated rats and mice in which spleen size was 3–5 fold larger when compared to control animals ($p<0.05$). Histological examination of liver and spleen did not reveal a cause for this change.

HBsAg and alpha-fetoprotein were detectable in serum of PLC/PRF/5 tumour-bearing nude mice and nude rats. As expected, much higher concentrations of these proteins were detected in nude mice, who have a small blood volume when compared with nude rats. HBsAg and AFP concentrations in the serum of athymic mice peaked at 2500 ng/ml and 350 µg/ml respectively when tumour weight was 2.3 g. Maximal HBsAg and AFP concentration in nude rats was only 370 ng/ml and 28 µg/ml respectively when tumour weight was 7–10 g. Alpha fetoprotein was also present in serum in Hep G2 tumour bearing mice. Both HBsAg and AFP were undetectable in serum of Sk-Hep 1 or Mahlavu tumour-bearing mice or rats. In every case in which HBsAg and AFP were detected in the serum, they were also present in tumour

Table 4. Tumourigenicity of human hepatoma cells in nude mice

Hepatoma cell line injected	No. inj. cells $\times 10^6$	Treatment[a]	Latency (days)	Tumour frequency: No. of mice with neoplasm / No. of mice injected	Mean tumour weight (g)
PLC/PRF/5	1–3	None	–	0/8	–
	4–5	None	21–34	10/24	–
	7–10	None	14–23	9/12	1.3
	2–3	X-irrad	14–24	3/9	–
	4–5	X-irrad	10–21	20/20	2.7
Sk-Hep 1	1	None	29	1/6	–
	2	None	20–22	2/4	–
	3	None	19–25	4/4	–
	4–5	None	17–21	5/6	0.22
	1	X-irrad	21–26	3/6	–
	2	X-irrad	17–21	5/6	–
	3–5	X-irrad	14–22	9/10	0.52
Mahlavu	1–3	None	–	0/8	–
	4–5	None	–	0/6	–
	7–10	None	50	1[b]/10	–
	20	None	45–48	2[b]/8	0.09
	1–3	X-irrad	–	0/6	–
	4–5	X-irrad	–	1/6	–
	7–10	X-irrad	35	4/10	0.5
Hep G2	1–20	None	–	0/12	–
	20	X-irrad	60–90	2/6	0.06

[a] *X-irrad:* mice irradiated with 400 rads 10 days before tumour cell injection
[b] tumours regressed
Animals were sacrificed 3 weeks after tumour detection except for Hep G2 bearing mice which were sacrificed at 7 weeks

tissue of the same animals as determined by immunohistological staining (data not shown). Thus HBsAg was present in over 50% of tumour cells, while AFP usually appeared in small clusters throughout the tumour tissue (Table 2). Neither HBeAg, anti-HBe, anti-HBc nor anti-HBs were detected in serum of 30 PLC/PRF/5 tumour bearing irradiated mice and rats.

Discussion

Relevant model systems are needed to assess the response to newly designed modes of treatment for human HCC. In contrast with the straightforward induction of chemical hepatocarcinogenesis in immunocompetent rodents, growth or human hepatoma tumours in immunodeficient animals is a more tedious task. Several hepatoma cell lines of human origin may now be utilized for the generation of human HCC tumours in athymic rodents. Historically, 2 of the 4 human hepatoma cell lines used to produce tumours in athymic rodents in the present study (PLC/PRF/5 and Mahlavu) were derived from HCC patients who were HBsAg carriers. Both cell lines are "persistently infected" with HBV and contain integrated HBV DNA [Shouval et al. 1981; D. Shouval et al., Hepatology, 4:1089, 1984, (abs)], but only PLC/PRF/5 cells synthesize and secrete HBsAg. Sk-Hep 1 and Hep G2 cells are devoid of HBV DNA.

Morphologically, the 4 human hepatoma cell lines represent a spectrum of tumour types encountered in clinical practice (Lapis 1983). In the athymic rodents, PLC/PRF/5 and Hep G2 hepatoma cells form well differentiated tumours consisting of polygonal cells. Tumour cell arrangement is in solid or trabecular patterns. In contrast, Sk-Hep 1 and Mahlavu tumours consist of poorly differentiated, spindle-shaped carcinoma cells. The 4 types also differ in the amount and composition of stroma. PLC/PRF/5 and Hep G2 nude rodent derived tumours have a relatively scanty stroma, while Sk-Hep 1 and Mahlavu tumours are rich in intercellular connective tissue fibers. A unique quality of both PLC/PRF/5 and Hep G2 tumours is the presence of scattered spaces, often lined by tumour cells but occasionally with endothelium. These spaces (which were sometimes also recognized macroscopically) show a strong resemblance to peliosis hepatis (Zak 1950), the significance of which is not clear. The emergence of such spaces may be related to the outgrowth of the host blood

supply leading to necrosis, which is considered to be one of the aetiologic factors for peliosis (Kintzen and Silney 1960; Zak 1950).

The morphological differentiation of the 4 tumour types parallels their functional differentiation. PLC/PRF/5 and Hep G2 cells are well differentiated polygonal cells that, in addition to their microscopic resemblance to normal hepatocytes, have retained their capacity to synthesize and secrete a variety of liver derived proteins (Aden et al. 1979; Knowles and Aden 1980; Shouval et al. 1981), including alpha-feto protein (Bassendine et al. 1980; Knowles 1980; Shouval et al. 1981). In contrast, the spindle-shaped Sk-Hep 1 and Mahlavu cells are morphologically as well as functionally distinct from hepatocytes. However, evidence for their hepatic origin is derived from the fact that both these cell lines express several hepatoma associated proteins (Carlson et al. 1985; Shouval et al. 1985). Furthermore, Mahlavu cells contain integrated HBV DNA and Sk-Hep 1 cells express a liver specific protein (LSP) also present in PLC/PRF/5 cells (Chisari et al. 1981). Ultrastructural analysis revealed the presence of cell junctions in all 4 tumour cell types, thus confirming the epithelial origin of these lines. In addition, microvilli were identified in PLC/PRF/5 cells, while structures resembling bile canaliculi were present in 3/4 tumour types.

One of the interesting findings in the present study is the significant difference in tumourigenicity of the various hepatoma cell lines in athymic mice and rats. A wide variety of human tumours grow readily in athymic nude mice and to a somewhat lesser extent in the nude rats (Brooks et al. 1980; Colston et al. 1981; Colston et al. 1982; Festing et al. 1978; Fogh and Orefeo 1977b). Indeed, we were able to grow the 4 hepatoma cell lines as s.c. tumours in athymic mice and excluding Hep G2, in athymic rats. However, tumourigenicity was lower in athymic rats and higher inoculation densities of tumour cells were required to establish growth when compared with athymic mice. Furthermore, established Sk-Hep 1 and Mahlavu tumours in the nude rat had a tendency to regress within 30–50 days of their appearance. This phenomenon of lower tumourigenicity of human carcinoma cell lines in athymic rats when compared with nude mice has been previously observed using a variety of human cell lines (Colston et al. 1981; Colston et al. 1982; Festing et al. 1978). The higher resistance of the congenitally athymic rats to acceptance of xenografts has been linked to an enhanced NK cell activity. Unlike the situation in the nude mouse, the NK cell activity is not age dependent in the nude rat (Brooks et al. 1980; Lotzova et al. 1984). The tumourigenicity of hepatoma cell lines in nude rats was markedly enhanced following irradiation, thus implicating a radiation-sensitive host defense mechanism as responsible for resistance to tumour growth. Some similarities may exist with mechanisms that control tumourigenicity of PLC/PRF/5 cell lesions in nude mice (Shouval et al. 1983). We have previously shown that spleen-derived NK cells from nude mice cause PLC/PRF/5 hepatoma cell lysis in vitro through an interferon regulated mechanism that is probably unrelated to HBsAg expression (Shouval et al. 1983). Interestingly, Mahlavu cells which are persistently infected with HBV but do no express HBsAg had a very low tumourigenicity in non-irradiated animals. Therefore, hepatoma cell surface proteins, unrelated to HBsAg, may be responsible for generation of increased resistance of nude mice and rats to HCC cells. In this context it is interesting to note that Mahlavu and Sk-Hep 1 tumours caused significant splenomegaly in host animals.

There are marked differences of tumourigenicity among the hepatoma cell lines within the same species. One possible factor that may influence its extent is the degree of differentiation. Human carcinoma cell lines do not usually produce metastases in the nude mouse (Giovanella 1978). In contrast with a previous report (Sattler 1982), we detected a single mouse in which pulmonary metastases were present following s.c. tumour cell injection out of 100 injected with PLC/PRF/5 cells. No metastases were observed in Hep G2, Sk-Hep 1 or Mahlavu tumour-bearing mice. In athymic rats only 1/42 PLC/PRF/5 tumour-bearing animals and 1/29 Sk-Hep 1 tumour-bearing rats showed pulmonary metastases. Therefore spontaneous metastases of human hepatoma cells in athymic rodents is a rare and unpredictable event.

Finally, it seems that nude mice, which have a smaller blood volume and harbor less necrotic tumour tissues, are suitable hosts for short range testing of experimental chemo- and immunotherapy. In contrast, tumour bearing nude rats which are more resistant to infections may be maintained without laminar flow hoods for periods of 1–2 years and are more suitable for long term experiments.

Acknowledgement. This study was supported by grants from the R. Molin Memorial Foundation, the US – Israel Binational Science Foundation grant no 3158/84, The National Cancer Institute and the US National Institutes of Health, Grants AcS 439, CA 30017. Part of these studies was performed by D.S. while on leave as an NIH Fogarty International Research Fel-

low at the Liver Research Center, Albert Einstein College of Medicine, Bronx, NY, USA.

The authors thank Dr. C. Hanson for his generous support in establishing the breeding colony of nude rats and Dr. B. Knowles for providing the Hep G2 cells. We are grateful to Dr. Hans Popper for reviewing the manuscript and to Dr. L. Biempica for advice. The technical assistance from Mrs. R. Adler, M. Anton, E. Hurston and M. Richardson is much appreciated.

References

Aden DP, Fogel A, Plotkin S, Damjanov I, Knowels BB (1979) Controlled synthesis of HBsAg in a differentiated human liver carcinoma cell line. Nature 282:615–616

Alexander JJ, MacNab G, Saunders R (1978) Studies on in vitro production of hepatitis B surface antigen by a human hepatoma cell line. Perspec Virol 10:103–117

Bassendine MF, Arborgh BAM, Shipton N, Monjardino J, Aranguibel F, Thomas HC, Sherlock S (1980) HBsAg and alpha feto protein secreting human primary liver cell cancer in athymic mice. Gastroenterology 79:528–532

Brooks CG, Webb PJ, Robins RA, Robinson G, Baldwin RW, Festing MFW (1980) Studies on the immunobiology of rnu/rnu nude rats with congenital aplasia of the thymus. Eur J Immunol 10:58–65

Carlson RI, Ben Porath E, Shouval D, Strauss W, Isselbacher KJ, Wands JR (1985) Antigenic characterization of human hepatocellular carcinoma. Development of in vitro and in vivo immunoassays that use monoclonal antibodies. J Clin Invest 76:40–51

Chang R (1954) Continuous subcultivation of epithelial like cells from normal human tissue. Proc Soc Exp Biol NY 87:440–443

Chen JN (1964) Establishment in vitro and some preliminary observations on a strain of human liver cell carcinoma. Acta Unio Int Contra Carcinoma 20:1314–1315

Chisari FV, Bieber MS, Johnson CA, Xavier C, Anderson DA (1981) Functional properties of lymphocyte subpopulations in hepatitis B virus infection. J Immunol 126:45–49

Colston MJ, Fieldsteel AH, Dawson PJ (1981) Growth and regression of human tumour cell lines in congenitally athymic (rnu/rnu) rats. JNCO 66:843–847

Colston MJ, Fieldsteel AH, Lancaster RD, Dawson PJ (1982) Immunologic studies and growth of human tumours in the athymic rat. In: Proceedings of the 3rd International Workshop on Nude Mice, Fischer, New York, pp 189–196

Das PK, Nayak NC, Tsiquaye KN, Zuckerman AJ (1980) Establishment of a human hepatocellular carcinoma cell line releasing hepatitis B virus surface antigen. Br J Exp Pathol 61:648–654

Doi I, Namba M, Sato J (1975) Establishment and some biologic characteristics of human hepatoma cell lines. Gann 66:385–392

Festing MFW, May D, Connors TA, Lowell D, Sparrow S (1978) An athymic nude mutation in the rat. Nature 274:365–366

Fogh J, Wright WC, Loveless JD (1977a) Absence of HeLa cell contamination in 169 cell lines derived from human tumours. J Natl Cancer Inst 58:209–214

Fogh J, Fogh JM, Orefeo T (1977b) One hundred and twenty seven cultured human tumour cell lines producing tumours in nude mide. J Natl Cancer Inst 59:221–226

Giovanella BC, Stehlin JS, Williams LJ Jr, Lee SS, Shepard RC (1978) Heterotransplantation of human cancers into nude mice. Cancer 42:2269–2281

Habu S, Fukui H, Shinamura K, Kasai M, Nagai Y, Okumura K, Tamoki N (1981) In vivo effects of anti-asialo GMl. I. Reduction of NK activity and enhancement of transplanted tumour growth in nude mice. J Immunol 127:34–38

Hirohashi S, Shimosato Y, Kameya T, Koide T, Mukojima T, Taguchi Y (1977) Morphological and functional aspects of human liver cell carinomas transplanted in nude mice. Proc 2nd Int Workshop on nude mice, University of Tokyo Press, Tokyo, pp 427–434; G Fischer, Stuttgart

Hirohashi S, Shimosato Y, Kameya T, Koide T, Mukojima T, Taguchi Y, Keizo Kageyama (1979) Production of alpha feto protein and normal serum proteins by xenotransplanted human hepatomas in relation to their growth and morphology. Cancer Res 39:1819–1828

Kintzen W, Silny J (1960) Peliosis hepatis after administration of fluoxymesterone. Can Med Assoc J 83:860–862

Knowles BB, Adem DP (1980) Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science (USA) 209:497–499

Lapis K (1983) Morphologic expression of neoplasia in human and experimental liver tumours. In: 13th Int Cancer Congress, Part D, Research and Treatment, Riss, New York, pp 67–76

Lotzova E, Savary CA, Gray KN, Raulston GL, Jardine JH (1984) NK cell profile of two random bred strains of athymic rats. Exp Hematol 12:633–640

Macnab GM, Alexander JJ, Lecatsas G, Bey EM, Urabanowich JM (1976) Hepatitis B surface antigen produced by a human hepatoma cell line. Br J Cancer 34:509–515

McDowell EM (1978) Fixation and Processing. In: Trump BF, Jones RT (eds) Diagnostic electron microscopy. Whey and Sons, New York, 1:pp 113–119

Nakabayashi H, Takketa K, Yamane T, Miyazeke M, Miyano K, Sato J (1984) Phenotypical state of a human hepatoma cell line HuH-7 in long term culture with chemically defined medium. Gann 75:151–158

Owens R, Smith H, Nelson-Rees W, Springer EL (1976) Brief communication: Epithelial cell cultures from normal and cancerous human tissues. J Natl Cancer Inst 56:843–846

Prozesky OW, Brits CJ, Grabow WOK (1972) In vitro culture of cell lines from Australia antigen positive and negative patients. In: Saunders SJ, Terblanche J (eds) Liver, Pitman Medical Publications, London, pp 358–360

Reid LM, Minato N, Gresser I, Holland J, Kadish A, Bloom BR (1981) Influence of anti-mouse interferon serum on the growth and metastasis of tumour cells persistently infected with virus and of human prostatic tumours in athymic nude mice. Proc Nat Acad Sci USA 78:1171–1175

Sattler FR, Paolucci S, Kreider JW, Ladda RL (1982) A human hepatoma cell line (PLC/PRF/5) produces lung metastases and secretes HBsAg in nude mice. Eur J Cancer Clin Oncol 18:381–389

Shouval D, Reid LM, Chakraborty PR, Ruiz-Opazo N, Morecki R, Gerber MA, Thung SN, Shafritz DA (1981) Tumourigenicity in nude mice of a human hepatoma cell line containing hepatitis B virus DNA. Cancer Res 41:1342–1350

Shouval D, Shafritz DA, Zurawski VR Jr, Wands JR (1982a) Immunotherapy in nude mice of a human hepatoma using monoclonal antibodies against hepatitis B virus. Nature (Lond) 298:567–569

Shouval D, Wands JR, Zurawski VR Jr, Isselbacher KJ, Shafritz DA (1982b) Protection against experimental hepatoma formation in nude mice by monoclonal antibodies to hepatitis B virus antigen. Hepatology 2:128s–133s

Shouval D, Rager-Zisman B, Quan P, Shafritz DA, Bloom BR, Reid LM (1983) Role in nude mice of interferon and NK cells in inhibiting the tumourigenicity of human hepatocellular carcinoma cells infected with heaptitis B virus. J Clin Invest 72:707–717

Shouval D, Eilat D, Carlson RI, Adler R, Livni N, Wands JR (1985) Human hepatoma associated cell surface antigen: Identification and characterization by means of monoclonal antibodies. Hepatology 5:347–356

Thomas HC, Montano L, Goodall A, de Koning R, Oledapo J, Wiedman KH (1982) Immunological mechanisms in chronic hepatitis B virus infection. Hepatology 2:116s–121s

Watanabi T, Morizane T, Tsuchimotok K, Inagaki Y, Munakata Y, Nakamura T, Kumagai N, Tsuchiya M (1983) Establishment of a cell line (HCC-M) from a human hepatocellular carcinoma. Int J Cancer 32:141–146

Zak FG (1950) Peliosis hepatis. Am J Pathol 26:1–15

Accepted November 25, 1987