Modification Form for Permit BIO-RRI-0056

Permit Holder: Wei-Yang Lu

Approved Personnel
(Please stroke out any personnel to be removed)

Shuanglian Wang

Yun-Yan Xiang

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. Give the full name - do not abbreviate.
Approved Microorganisms		
Approved Primary and Established Cells	[Estabslihed] (Human): A549. BEAS-2B, IB3-1, IB3-837, (Human): Hep G2. (Rats): Clone 9.	MZ-ChA-1 cell line
Approved Use of Human Source Material		
Approved Genetic Modifications (Plasmids/Vectors)		
Approved Use of Animals	C57BL/6, BAL B/C Mice.	
Approved Biological Toxin(s)		

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF..*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: Weijang Lu

Current Classification: 2 **Containment Level for Added Biohazards:**

Date of Last Biohazardous Agents Registry Form: Nov 18, 2009

Date of Last Modification (if applicable): Feb 26, 2010

BioSafety Officer(s):

Chair, Biohazards Subcommittee: **Date:**

Brief description of the work being done with Mz-ChA-1 cell line

By Wei-Yang Lu

The cell line we want to use is human biliary epithelial cell line Mz-ChA-1. **We will perform patch-clam recordings in the cells**.

We have found that in rodents such as rat and mouse the liver bile duct epithelial cells express A-type γ-aminobytryic acid receptors ($GABA_AR$). This finding is novel and interesting because GABA is a neurotransmitter in the brain, and confirming the expression of this neurotransmitter receptor in human biliary epithelial cells bear biomedical significance.

$GABA_ARs$ are anionic channels. We will make patch-clamp recording in the cells to examine whether the human biliary epithelial cell line Mz-ChA-1 express functional $GABA_ARs$. If so, we will use the cell to study the role of $GABA_AR$ in the regulation of bile production.

Information of Mz-ChA-1 cell line (also see the attached paper)

Mz-ChA-1 was derived from an abdominal wall metastasis of a highly differentiated papillary adenocarcinoma of the gall bladder of a 55-year-old female patient.

Tissue specimens were minced and teased with fine scissors in petri dishes, under sterile conditions, in a few drops of phosphate-buffered saline (PBS) to which 100 IU/ml penicillin and 100/zg/ml streptomycin had been added. After 2 washes in this solution and removal of larger tissue clumps (> 1 mm3), 2 ml of the remaining cell suspension was gently centrifuged at 150 x g for 5 min at room temperature; 2 ml of approximately 5 x 105 living cells/ml (trypan blue dye exclusion test) were placed in 75-cm 3 tissue culture flasks (Nunc, Roskilde, Denmark) in CMRL-medium (GIBCO, NY, U.S.A.) supplemented with penicillin 100 IU/ml, streptomycin 100/~g/ml, and 15% prescreened fetal bovine serum (FBS, heat-inactivated at 56 °C for 30 rain). Tissue cultures were kept at 37 °C in a 5% CO 2 atmosphere. Initially 50% of the culture medium was replaced every 2 or 3 days. Islands of epithelial cells could be clearly distinguished from surrounding fibroblasts after 2 weeks in culture. Cell cultures were subcultured by light trypsin treatment (0.25% in Ca 2^+- and $Mg2^+$-free PBS) until cell islands detached.

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Biliary Adenocarcinoma

Characterisation of Three New Human Tumor Cell Lines

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Summary

Three human cell lines from adenocarcinomas of the extrahepatic biliary tract were established in permanent tissue culture. Mz-ChA-1 and Mz-ChA-2 were cultured from mechanically dissociated gallbladder adenocarcinoma metastases and SK-ChA-1 was grown from malignant ascites of a patient with primary adenocarcinoma of the extrahepatic biliary tree. Cell doubling times in tissue culture are 3–4 days for Mz-ChA-1 and approximately 2 days for Mz-ChA-2 and SK-ChA-1. All three tumour cell lines were successfully transplanted to nude mice, inducing progressive tumour growth. Histologically, nude mouse tumours resembled the original adenocarcinomas. In vitro formation of gland-like structures were regularly seen in Mz-ChA-1 and Mz-ChA-2 but only occasionally in SK-ChA-1. All three cell lines formed contacts through interdigitating processes with desmosomes and junctional complexes. On scanning electron microscopy, an abundance of microvilli was seen at the cell surfaces. Chromosome analyses of all three tumour cell lines showed a wide range of numerical abnormalities and presence of marker chromosomes. Mz-ChA-1 appears to be highly differentiated with cells producing mucus. Mz-ChA-2 synthesizes components of complement C2, C3 and C5, while Mz-ChA-1 and SK-ChA-1 produce only C3 in detectable quantities. In addition, Mz-ChA-2

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supernatants are positive for ferritin and α_1-fetoprotein, but not CEA; while Mz-ChA-1 and SK-ChA-1 produce only CEA. Supernatants of all three cell lines are positive for N-acetyl neuraminic acid (NANA), phosphohexoisomerase (PHI) and LDH, and negative for α_2-macroglobulin, α_1-anti-trypsin, γ-GT, AP, coeruloplasmin, haptoglobin and albumin. A high cloning efficiency renders these new tumour cell lines suitable for continued studies on clonal heterogeneity in malignant tumours. The establishment of these cell lines in tissue culture facilitates further studies on the biology of upper gastrointestinal tract cancer in man.

Introduction

Biliary adenocarcinoma is an uncommon cancer that is often discovered accidentally at the time of cholecystectomy for cholelithiasis. In women, about 67% are gallbladder carcinomas, while in men bile duct cancers account for 60% [1].

The aetiology and pathogenesis are poorly understood, but gallstones and chronic cholecystitis are considered major risk factors. An association with ulcerative colitis has been suggested, but statistically not established [2]. Liver-fluke infections are known promoters of biliary adenocarcinomas, possibly in association with dietary nitrosamine uptake [3].

As effective chemotherapy is unavailable to date, any chance of a cure through adequate surgery depends on early detection.

To date, adenocarcinoma cell lines originating from the gallbladder or extrahepatic biliary tract have rarely been established in permanent tissue culture [4–6] and little is known about their biology or biochemical markers. Tumour cell lines in permanent tissue culture open possibilities to further define markers that may become clinically relevant [7–12].

Additionally, tissue culture cell lines facilitate studies on clonal tumour heterogeneity, and may thus add to a further understanding of the role of the immune response to cancers.

We report here on three newly established adenocarcinoma cell lines derived from two carcinomas of the gallbladder and from a primary carcinoma of the extrahepatic biliary tract, with emphasis on cell morphology, differentiation, chromosomal heterogeneity and biochemical markers.

Methods

Patients

Mz-ChA-1 was derived from an abdominal wall metastasis of a highly differentiated papillary adenocarcinoma of the gall bladder of a 55-year-old female patient. Ten years earlier the patient had been treated for cholecystitis. Five months before the tumour specimen was obtained for tissue culture, a cholecystectomy was performed because of symptomatic cholelithiasis. The surgical specimen showed the unexpected presence of a highly differentiated, mucus producing gallbladder adenocarcinoma. Subsequently, re-laparotomy became necessary due to obstructive

jaundice from spread along the ligamentum gastrocolicum, the bulbus duodeni, the proximal duodenum and the abdominal wall. After percutaneous transhepatic drainage of the biliary system, jaundice subsided. Serum levels of CEA were slightly elevated around 20 ng/ml with AFP levels in the normal range. After three courses of sequential combination chemotherapy with cisplatinum and etoposide, no objective tumour response was seen. Treatment was discontinued because of rapid deterioration of the patient, who died a few days later.

Mz-ChA-2 was derived from liver metastases of a less differentiated adenocarcinoma of the gallbladder of a 63-year-old female patient. She had a 3-year history of chronic cholecystitis due to a solitary stone in the gallbladder. Cholecystectomy and right dorsolateral hemihepatectomy were performed for treatment of local tumour spread to the liver.

Serum levels of CEA were found to be normal; AFP was not measured at that time. Ferritin levels, though, were found to be elevated to 319 ng/ml. No isoferritin patterns were determined. Postoperatively, the patient remained in remission for 13 months; but the tumour recently recurred with an intrahepatic mass considered to be metastatic.

SK-ChA-1 was derived from malignant ascites of a 47-year-old female patient with a widespread undifferentiated adenocarcinoma originating from the extrahepatic biliary tree involving the common bile duct, the cystic duct and part of the body and bed of the gall bladder. Hepatic metastases, infiltration of the head of the pancreas and diffuse peritoneal carcinomatosis were present when the patient first presented with jaundice and massive ascites. Palliative intraabdominal 5-fluorouracil instillations were given. The patient succumbed one month later.

Tissue culture

Tissue specimens were minced and teased with fine scissors in petri dishes, under sterile conditions, in a few drops of phosphate-buffered saline (PBS) to which 100 IU/ml penicillin and 100 μg/ml streptomycin had been added. After 2 washes in this solution and removal of larger tissue clumps (> 1 mm^3), 2 ml of the remaining cell suspension was gently centrifuged at $150 \times g$ for 5 min at room temperature; 2 ml of approximately 5×10^5 living cells/ml (trypan blue dye exclusion test) were placed in 75-cm^3 tissue culture flasks (Nunc, Roskilde, Denmark) in CMRL-medium (GIBCO, NY, U.S.A.) supplemented with penicillin 100 IU/ml, streptomycin 100 μg/ml, and 15% prescreened fetal bovine serum (FBS, heat-inactivated at 56 °C for 30 min). This culture medium was used throughout the study. Tissue cultures were kept at 37 °C in a 5% CO_2 atmosphere. Initially 50% of the culture medium was replaced every 2 or 3 days. Islands of epithelial cells could be clearly distinguished from surrounding fibroblasts after 2 weeks in culture. Cell cultures were subcultured by light trypsin treatment (0.25% in Ca^{2+}- and Mg^{2+}-free PBS) until cell islands detached.

Malignant ascites fluid was centrifuged, adjusted to approximately 5×10^5 living cells/ml and cultured as cell suspensions obtained from solid tumour specimens.

All cultures were regularly tested for fungal, bacterial and mycoplasma infections. Infected cultures were discarded.

Phase contrast microscopy

Tissue culture flasks were routinely monitored under an inverted photomicroscope using phase contrast (Zeiss, Oberkochen, F.R.G.).

Transmission electron microscopy

For transmission electron microscopy, the tumour cells grown on cover glasses were fixed for 20 min in 2.5% phosphate-buffered glutaraldehyde solution (pH 7.3) and post-fixed for 30 min in 1% OsO_4-solution. After embedding in Epon, thin sections were cut and stained with uranyl acetate and lead citrate. Electron micrographs were taken with an EM 301 (Philips) transmission electron microscope.

Scanning electron microscopy

For scanning electron microscopy, the tumour cells were fixed for 60 min in 2.5% phosphate-buffered glutaraldehyde solution (pH 7.3) and post-fixed for 60 min in 2% OsO_4-solution. After dehydration in ascending acetone series, the tumour cells were dried by the critical point method and sputtered with gold. Electron micrographs were taken with the PSEM (Philips) scanning electron microscope.

Growth kinetics and cloning efficiency

Growth kinetics were determined by seeding a fixed number of living cells into 5 parallel tissue culture flasks at day 0. Every 24–48 h one tissue culture flask was trypsinized and cells counted with a haemocytometer. Cloning efficiency was determined in 96-well flat bottom plates (Nunc, Roskilde, Denmark) by seeding 48 wells each at 30, 10, 3, 1, 0.3 cells/well and counting wells with growing cells after 2 weeks.

Heterotransplantation

Athymic mice (BALB/c, nu/nu) of both sexes at 5–6 weeks of age were inoculated subcutaneously with 1×10^7 cells in PBS in the lateral back. Tumour growth was assessed by taking 2 diameters of subcutaneous tumour implants at regular intervals of 3–4 days. Mice were kept in a pathogen-reduced laminar flow chamber.

Chromosome analysis

Based on growth characteristics and cell cycle duration, mitotic peaks were calculated and enhanced by feeding rhythm and by application of 0.5 μg colcemide/ml 4 h prior to harvesting. After a hypertonic shock of 11 min with 1% tri-sodium-citrate, cells were fixed with ethanol acetic acid at 3 to 1 and spread by an air-drying procedure for chromosome analysis.

Serological reagents

The following mouse monoclonal antibodies and polyclonal human antisera were used for serological testing (Table 1):

Serological procedures

Enzyme-linked immune sorbent assays (ELISA) with alkaline phosphatase

TABLE 1

Monoclonal antibody or polyclonal antiserum	Antigenic determinant	Reference or source of antibody
W6/32	HLA-A,B,C	ATCC[a] [15]
HB55	Ia monomorphic	ATCC [14]
13-17	Ia monomorphic	[14,15]
O5, Q14, M19, I12, L10	melanoma/melanocyte cell surface antigens	[11,16]
M221	blood group A	[8]
S8	blood group B	[8]
Anti-A	blood group A	Behringwerke AG,
Anti-B	blood group B	Marburg, F.R.G.

[a] American Type Culture Collection (ATCC), Rockville, MD, U.S.A.

(ALP) have been carried out according to a protocol developed by B. Doerken (personal communication). Briefly, microtest plates (Falcon, No. 3034) were seeded with 300 living cells/well and incubated over night at 37 °C, 5% CO_2 atmosphere to allow for attachment of cells. Cells were fixed with 0.025% glutaraldehyde in PBS at room temperature for 10 min, washed 3 times in PBS and covered with 10 ml of gelatine, 2 mg/ml in PBS for later use. Tests were performed with washed plates. Incubation of 5 μl of antibody at room temperature for 30 min was followed by incubation with 5 μl of goat-anti-mouse-IgG/alkaline phosphatase or goat-anti-human-Ig/alkaline phosphate (Sigma) 1:20 in PBS for 30 min with washes in between (3 × Tris–HCl, pH 7.6, 1 × Tris-buffer). Then, rabbit anti-goat-IgG/alkaline phosphatase (Sigma) 1:20 was added for 30 min. After 15 min incubation time with a naphthol-AS-BI-phosphate/fast-red-TR-salt solution (Sigma) and after washing of plates, target cells were scored (positive = red color reaction). For positive controls cell lines known to react with these antibodies were used (hypernephroma cell lines for blood group reagents, melanoma cell lines for the other monoclonal antibodies tested). As negative controls target cells were incubated with second antibody only, omitting the relevant monoclonal antibody or polyclonal antiserum (like blood group A and B reagents). Plates were only considered evaluable when controls were clearly negative.

Markers

Biochemical markers were determined and quantitated with the following methods and commercially available test kits (Table 2):

Complement assays

C3 and C5 were tested antigenically with an ELISA method using a combination of monoclonal antibodies against human C3a and C3b for testing native C3, and a combination of monoclonal antibodies against human C5a and C5b for testing native C5. In both cases the monoclonal antibody against the small peptide was used in the solid phase, and the antibodies against the C3b/C5b parts of the molecule as an enzyme-labeled sandwich (Klos and Bitter-Suermann, manuscript in preparation). Purified human C3 and C5 according to Tack et al. [13] were used as refer-

TABLE 2

Biochemical markers	Source, test
Lactate dehydrogenase (LDH), phospho-hexoisomerase (PHI), γ-glutamyl transferase (γ-GT), alkaline phosphatase (AP)	Standard methods of the German Society of Clinical Chemistry
N-Acetyl neuraminic acid (NANA)	Enzymatic test (Kyokuto, Japan)
Ferritin	Ferrizyme Diagnostic Kit (Abbott Laboratories, Chicago, IL, U.S.A.)
Haptoglobin, coeruloplasmin, albumin	Laser-nephelometry (Behringwerke, Marburg, F.R.G.)
α_1-Antitrypsin, α_2-macroglobulin	Colorimetric test (Boehringer, Mannheim, F.R.G.)
Carcinoembryonic antigen (CEA)	CEA-EIA (Abbott Laboratories, Chicago, IL, U.S.A.)
α-Fetoprotein (AFP)	AFP-EIA (Abbott Laboratories, Chicago, IL, U.S.A.)

ence protein for calibration. C3 and C5 protein contents of the samples were calculated according to the C3 and C5 standard curves. For C2 determination a functional haemolytic assay was used according to published procedures [14].

Results

Morphology

Phase contrast microscopy of cultured tumour cells showed adherent cell growth of typical epithelial cell types in all three tumour cultures. Single cells were polygonal or spindle-shaped varying greatly in size. Mz-ChA-1 and Mz-ChA-2 regularly formed gland-like structures in tissue culture, SK-ChA-1 only rarely. SK-ChA-1 appeared least differentiated, growing as single adherent cells or in small clusters. In contrast, Mz-ChA-1 and Mz-ChA-2 grew in cell clusters or clumps (Fig. 1b, 2b, 3b), and were difficult to dissociate enzymatically to form single-cell suspensions. On scanning electron microscopy of cultured cells an abundance of microvilli was seen on the cell surfaces (Figs. 1a, 2a, 3a). Contacts between single adjacent cells were formed through interdigitating processes and desmosomes in all three cell lines, and, additionally, through typical junctional complexes in Mz-ChA-1 and Mz-ChA-2, but not in SK-ChA-1, the least differentiated of these three adenocarcinoma cell lines (Figs. 1c, 2c, 3c). Cytoplasmic structures, as seen on transmission electron microscopy, were moderate numbers of mitochondria with irregular cristae, an abundance of rough endoplasmic reticulum, ribosomes and polysomes. Mz-ChA-1, showed signs of active mucous production with large intracytoplasmic droplets in many cells (Fig. 1c). In Mz-ChA-2, prominent bundles of filaments were detectable. No virus-like particles were seen in any of the three cell lines.

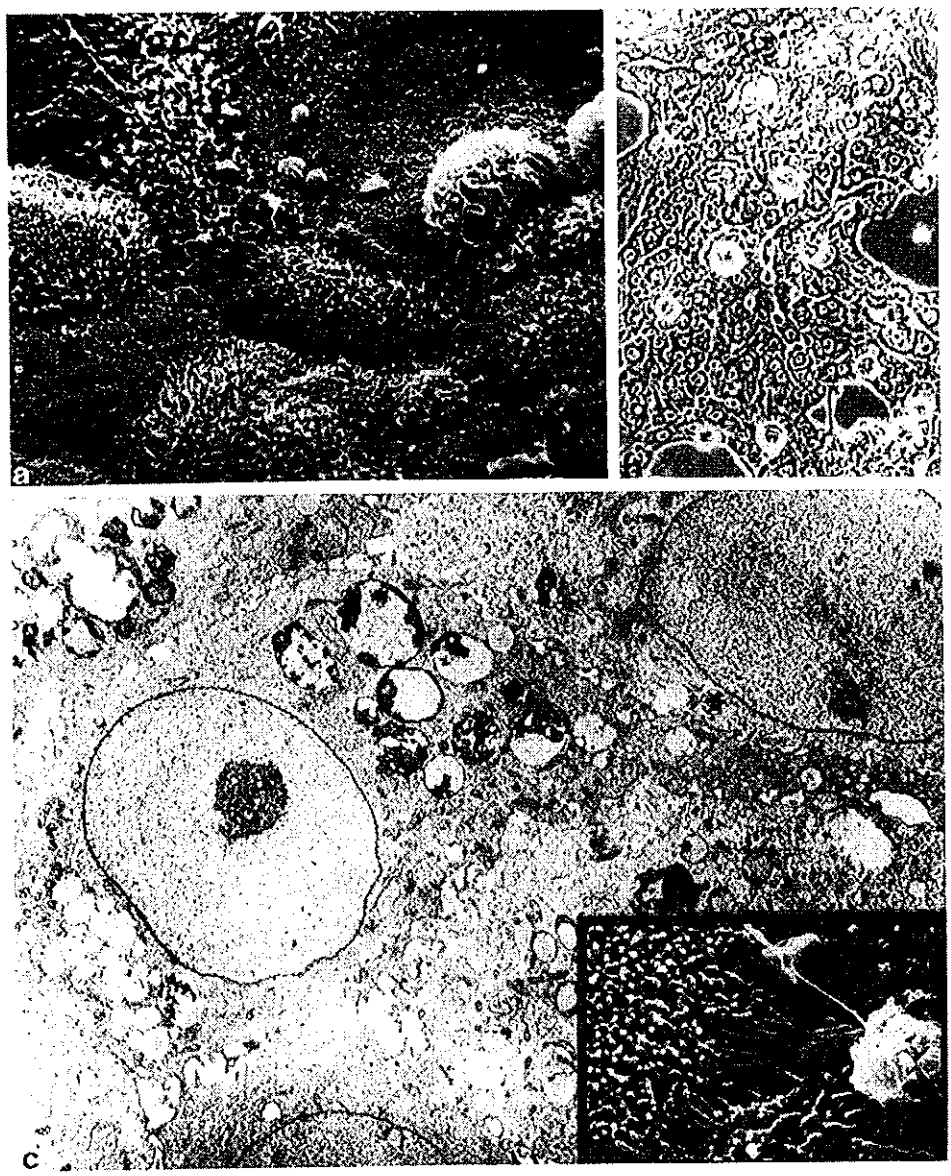


Fig. 1. Mz-ChA-1. *a:* Scanning electron micrograph showing numerous microvilli on the cell surface, × 10 500. *b:* Phase contrast photomicrograph; formation of gland-like structures by epithelial cells of polygonal and spindle shape with great variation in size; × 180. *c:* Intracytoplasmic mucus droplets are seen in transmission electron micrographs, × 5 500; cells are joined by interdigitating processes. Inset in *c*, × 25 000.

Growth kinetics and cloning efficiency

Cell doubling times around passage 10 were 3–5 days for Mz-ChA-1 and approximately 48–60 h for Mz-ChA-2. SK-ChA-1 doubled about every 48 h at passage 55–60.

The cloning efficiency in 96-well flat bottom plates is summarized in Table 3 at

various cell numbers seeded per well. The least differentiated cell line, SK-ChA-1, has the highest cloning efficiency with approximately 73% at one cell seeded per well.

Heterotransplantation

All three cell lines were successfully transplanted subcutaneously into nude mice. With an inoculum of 1×10^7 cells progressively growing tumours were regularly ob-

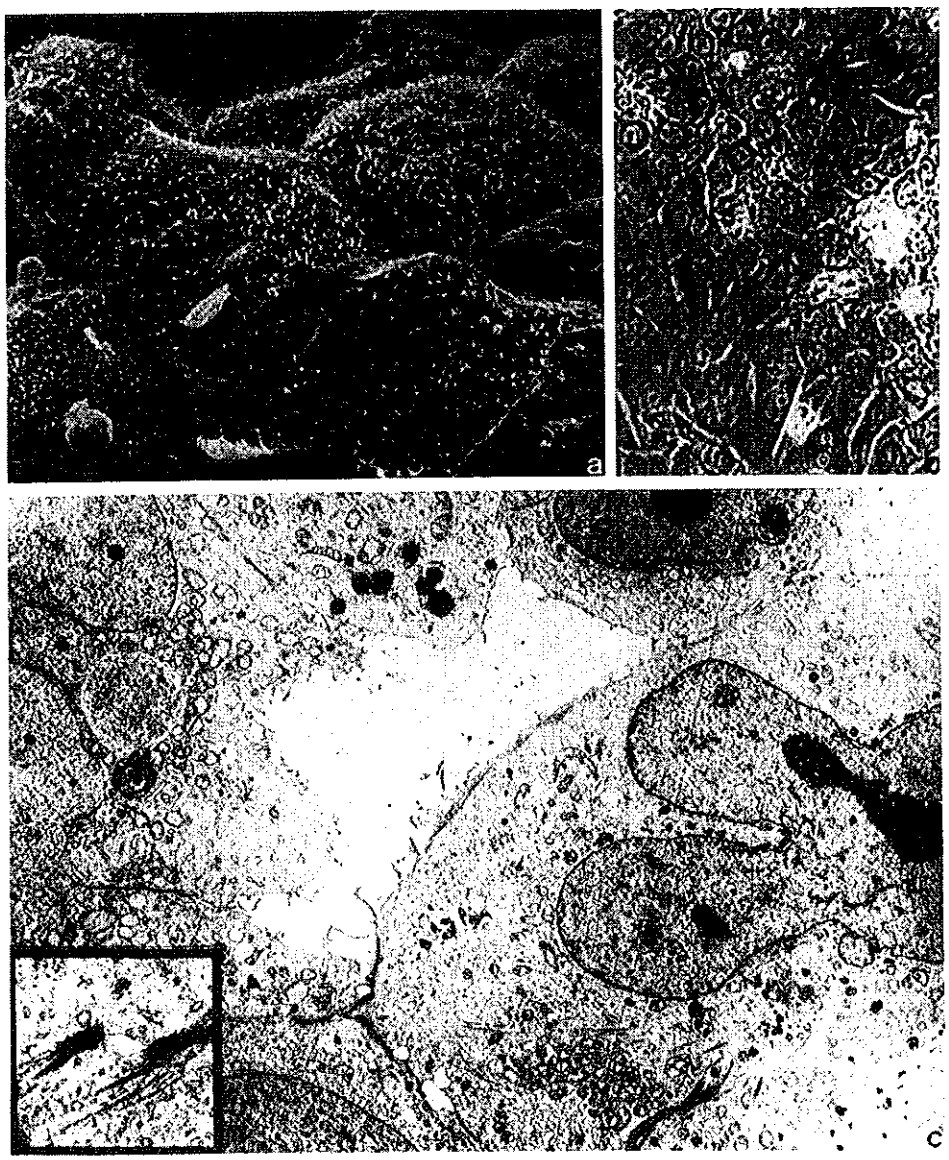


Fig. 2. Mz-ChA-2. *a:* In scanning electron micrographs microvilli are less numerous than in Mz-ChA-1, × 5 000. *b:* Phase contrast micrographs show similar cell shapes as in Mz-ChA-1, × 280. *c:* In transmission electron micrographs, gland-like structures with desmosomes (inset, × 13 800) and junctional complexes are seen; bundles of intracytoplasmic tonofilaments are present, × 3 400.

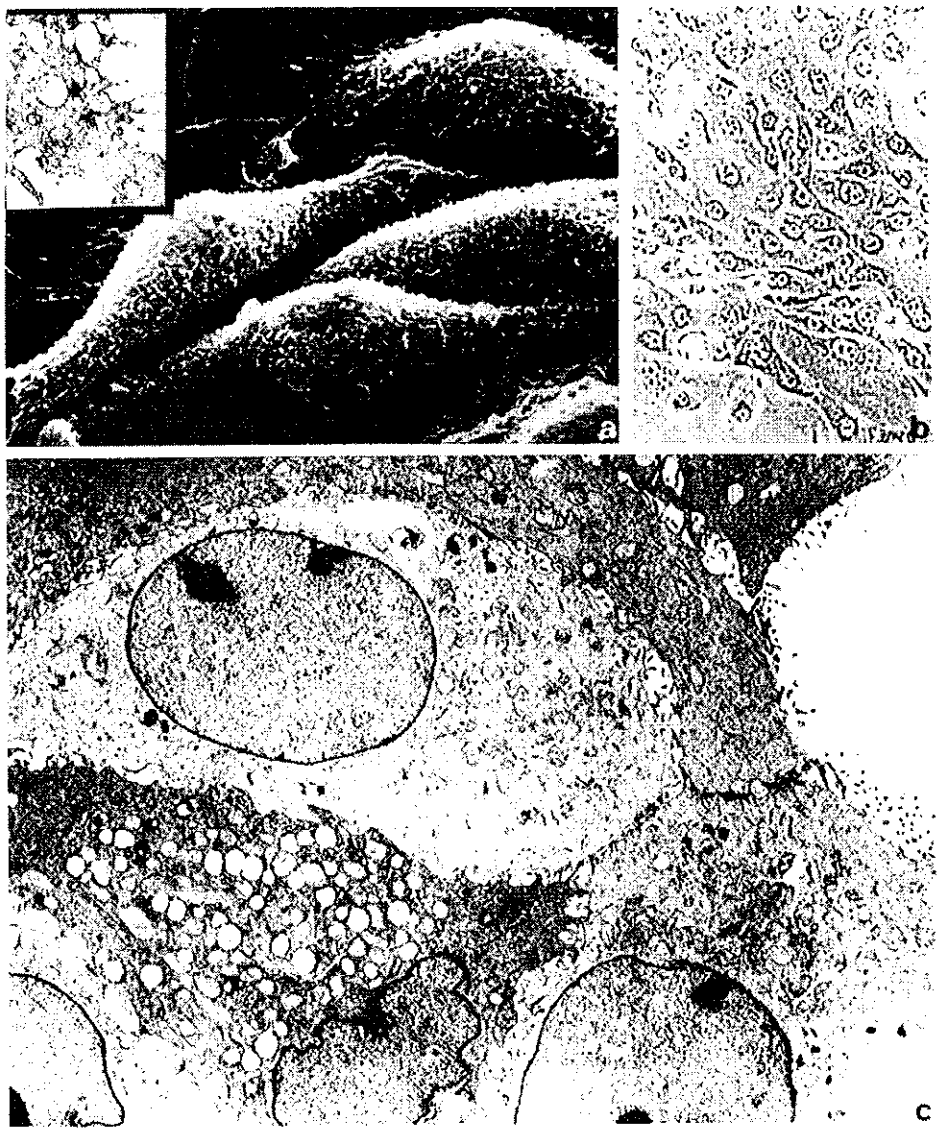


Fig. 3. SK-ChA-1. *a:* Microvilli and pseudopods of tumour cells can be identified in scanning electron micrographs, × 10 750. *b:* Phase contrast micrograph showing a monolayer of polygonal tumour cells, × 180. *c:* In transmission electron micrographs gland-like structures are rarely seen, × 3 800. Cell contacts are formed by interdigitating processes with desmosomes. Inset in *a*, × 9 100.

served. In Mz-ChA-1 and Mz-ChA-2 perpendicular diameters of subcutaneous tumour nodules doubled after approximately 14 days after an initial decline during the first 3 days after subcutaneous tumour injection. In SK-ChA-1 doubling times of subcutaneous tumour nodule diameters were 7–10 days. Light microscopy of these tumour specimens resembled the histomorphology of the tumours of origin in the patient. An example (Mz-ChA-1) is shown in Fig. 4a, b.

TABLE 3

CLONING EFFICIENCY OF BILIARY ADENOCARCINOMA CELL LINES IN 96-WELL PLATES

Cell line	Cells seeded per well	No. positive per No. seeded[a,b]	%
Mz-ChA-1	30	19/48	40
	10	11/48	23
	3	6/48	13
	1	1/48	2
	0.3	1/96	1
Mz-ChA-2	30	38/48	79
	10	30/48	63
	3	22/48	46
	1	4/48	8
	0.3	9/96	9
SK-ChA-1	10	30/30	100
	3	29/30	97
	1	22/30	73
	0.3	11/30	37

[a] Assayed after 14 days in culture.

[b] Representative examples of at least 2 independent experiments.

Chromosome analyses

Chromosome analyses in all three cell lines revealed a range of numerical abnormalities as well as structural abnormalities some of which are considered to be marker chromosomes. Variations in chromosome number are plotted in Fig. 5a, b, c, showing modal chromosome numbers of 70–89 with a range of 39–144 for Mz-ChA-1; Mz-ChA-2 has a modal chromosome number of 74–96 with a range of 61–194 showing few atypical double minutes; SK-ChA-1 has a modal chromosome number of 61–66 ranging from 61–164 with few single minutes and a dicentric large marker chromosome in some metaphases. As an example, a representative karyotype is shown in Fig. 6.

Cell surface antigen expression and biochemical markers

Table 4 summarizes the cell surface antigen phenotype of Mz-ChA-1, Mz-ChA-2, and SK-ChA-1 as determined by monoclonal antibodies defining various antigenic systems [7,11,12,14–16]. While HLA class I antigens are expressed on all three cell lines, HLA class II antigens are not detectable. Mz-ChA-2 is the only one cell line expressing gp 130, an antigen present on malignant melanoma cells, and also O_5, a heat-stable antigen not yet biochemically characterized. The antigens, gp 95, a transferrin-related molecule [17] and M 19, expressed on Mz-ChA-1, defining another heat-stable antigen originally found on malignant melanomas, are expressed on Mz-ChA-1 only. Blood group A-associated antigens are not detected on any of the three cell lines. Blood group B-associated antigens, though, are weakly expressed on Mz-ChA-1 only.

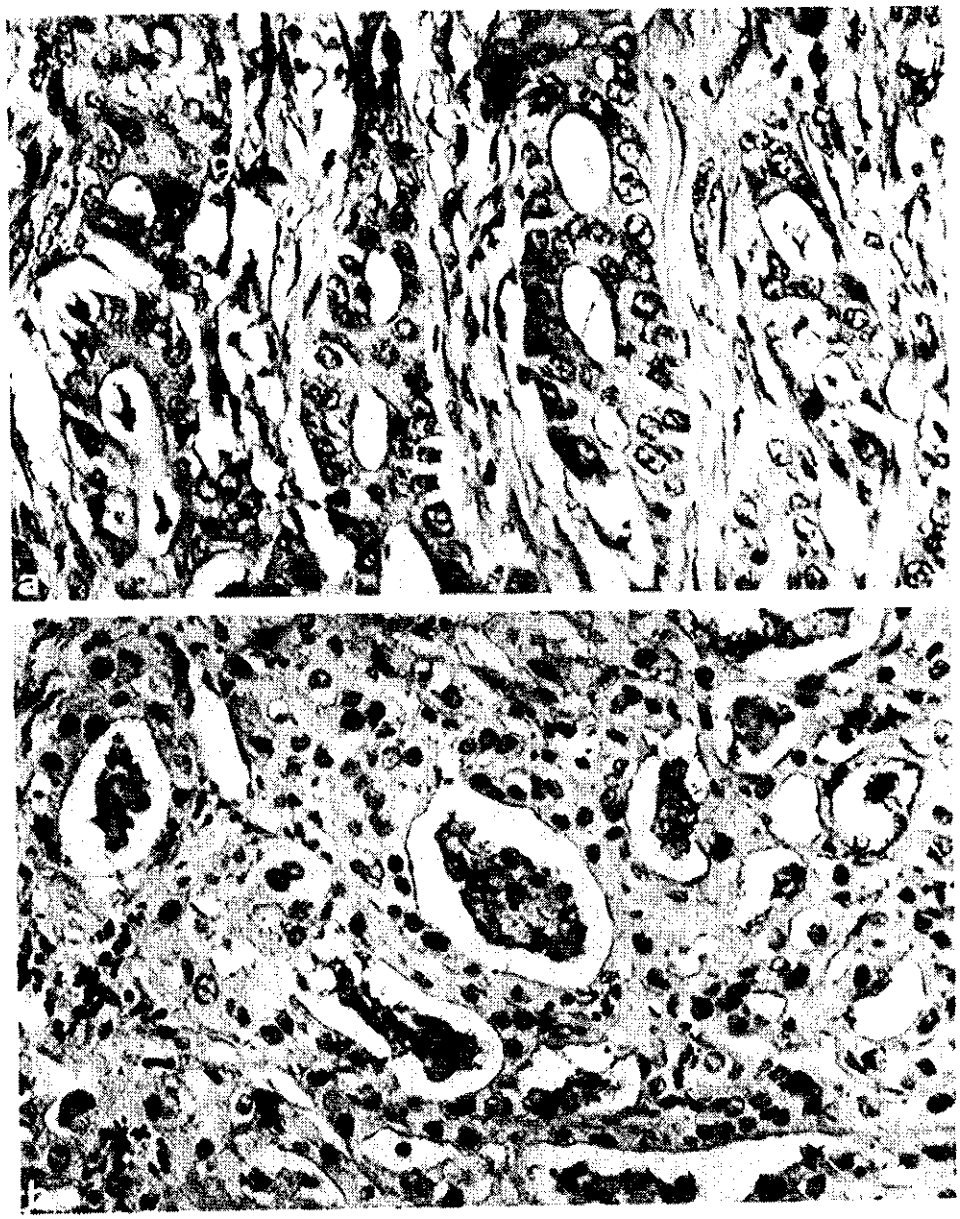


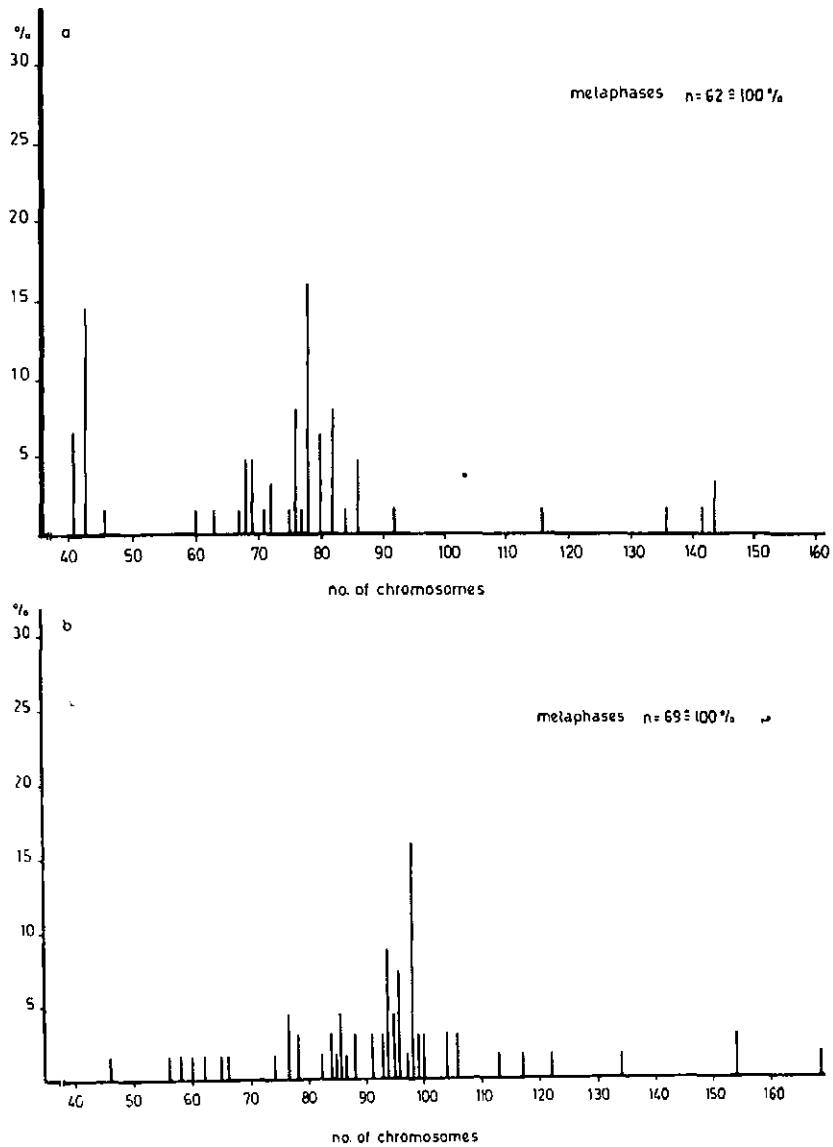
Fig. 4. Mz-ChA-1. *a:* Histologic features of the tumour specimen as obtained at surgery; H and E, × 370. *b:* SK-ChA-1 grown in a nude mouse for 6 weeks subcutaneously; H and E, × 370. Except for the stromal elements (nude mouse) both tumour specimens show a well differentiated adenocarcinoma.

In Table 5, biochemical markers detected in tissue culture supernatants of biliary adenocarcinoma cell lines are summarized. Large quantities of CEA are detectable in Mz-ChA-1 supernatants and small quantities in SK-ChA-1. Mz-ChA-2 is negative for CEA but produces large amounts of AFP, while the other two cell lines are negative for this marker. Marginal quantities of ferritin are only seen in Mz-ChA-2 supernatants. All three cell lines synthesize small or intermediate quantities of

NANA, PHI, and LDH, but no α_1-antitrypsin and α_2-macroglobulin, haptoglobin, albumin, coeruloplasmin, γ-GT or ALP.

Synthesis of components of the complement system (C2, 3, 5)

The third component of the complement system was detected in all three biliary adenocarcinoma cell lines as detected by antigen quantitation with monoclonal antibodies (Table 6). C5 and C2 are detectable only in supernatants of Mz-ChA-2. In this cell line C2 biosynthesis was quantitated over 48 h by harvesting culture medium every 8 h or every 24 h. The C2 presence was determined haemolytically (Fig. 7). According to these findings, 1×10^6 Mz-ChA-2 cells synthesize approximately 10×10^7 molecules of C2 every 8 h. Haemolytic activity in 24-h supernatants was



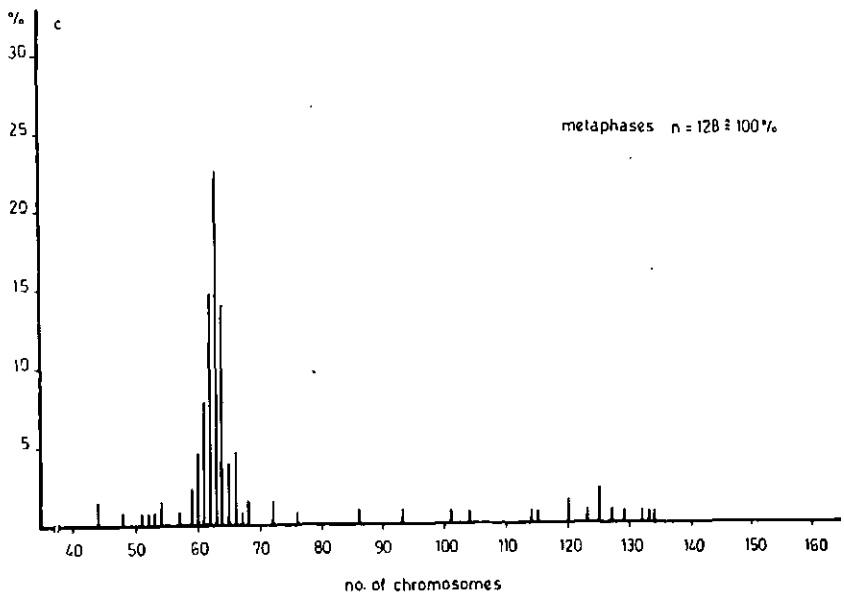


Fig. 5. Number of chromosomes is plotted against percent of metaphases evaluated: *a:* Mz-ChA-1, 62 metaphases; *b:* Mz-ChA-2, 69 metaphases; *c:* SK-ChA-1, 128 metaphases.

lower than calculated for accumulated 8-h supernatants, reflecting the short half life of functional C2.

Discussion

Cultured human cancer cells of various organ systems have greatly contributed to the understanding of tumour biology and the definition of tumour markers. Adeno-

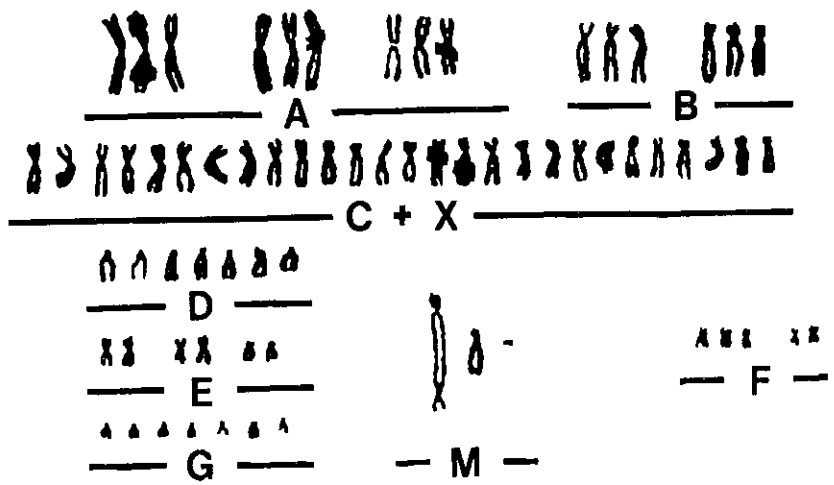


Fig. 6. Representative karyotype of SK-ChA-1 showing aneuploidy with trisomic elements and various marker chromosomes (M).

TABLE 4

CELL SURFACE ANTIGEN PHENOTYPE OF BILIARY ADENOCARCINOMA CELL LINES

	Antigen	Mz-ChA-1	Mz-ChA-2	SK-ChA-1
Monoclonal antibody				
W6/32	HLA-class I	+[a]	+	+
HB55	HLA-class II, (Ia) monomorphic	–	–	–
13-17	HLA-class II, (Ia) monomorphic	–	–	–
O_5	heat-stable antigen	–	+	–
Q_{14}	gp 130	–	+	–
M_{19}	heat-labile antigen	+	–	–
I_{12}	gp 95	+	–	–
L_{10}	gp 95	+	–	–
M221	blood group A	–	–	–
S8	blood group B	+	–	–
Polyclonal antibodies				
Anti-A	blood group A	–	–	–
Anti-B	blood group B	+	–	–

[a] –, +: Positive or negative staining in ALP-ELISA.

carcinomas of the biliary tract have rarely been established as permanent cell lines in tissue culture [4–6]. The 3 biliary adenocarcinoma cell lines described here are markedly different from one another. Morphology and growth patterns in vitro and in vivo after heterotransplantation to nude mice, demonstrate the malignant nature

TABLE 5

BIOCHEMICAL MARKERS SYNTHESIZED AND RELEASED INTO TISSUE CULTURE SUPERNATANT[a] OF BILIARY ADENOCARCINOMA CELL LINES

Biochemical markers	Units	Medium[b]	Mz-ChA-1	Mz-ChA-2	SK-ChA-1
CEA	ng/ml	0	50	0	7
AFP	ng/ml	0	0	2648	0
Ferritin	ng/ml	2	0	9	0
NANA	ng/dl	7	24	22	25
LDH	U/l	4	36	36	49
PHI	U/l	0	30	34	33
α_1-Anti-trypsin	U/ml	0.42	0.55	0.71	0.70
α_2-Macroglobulin	U/ml	1.2	1.55	1.6	1.4
Haptoglobin	mg/dl	0	0	0	0
Coeruloplasmin	mg/dl	0	0	0	0
Albumin	mg/dl	0	0	0	0
γ-GT	U/l	0	0	0	0
AP	U/l	0	0	0	0

Abbreviations: AFP = α-fetoprotein, AP = alkaline phosphatase, CEA = carcinoembryonic antigen, γ-GT = gamma-glutamyl transferase, LDH = lactate dehydrogenase, PHI = phosphohexoisomerase, NANA = N-acetylneuraminic acid.

[a] Supernatants (4 ml) of approximately 80% confluent tissue culture flasks (approx. 1×10^6 cell/flask) have been assayed 4 days after last change of tissue culture medium.

[b] Controls were fully supplemented tissue culture medium samples.

TABLE 6

SYNTHESIS OF THE 3rd AND 5th COMPONENT OF THE COMPLEMENT SYSTEM (C3, C5, ANTIGEN QUANTITATION)

Supernatants were harvested after 1 or 6 days without change of tissue culture media from approximately 80% confluent tissue culture flasks or a total cell number of about 1×10^6 cells per flasks.

Cell line	C3 (ng/ml)		C5 (ng/ml)	
	1 day	6 days	1 day	6 days
Mz-ChA-1	77	302	15	15
Mz-ChA-2	106	284	65	147
SK-ChA-1	111	315	15	15
Media control	14	14	15	15

of these cell cultures and establish their identity with the tumours of origin in the patients. Markers of epithelial differentiation are preserved to different degrees in these cell lines. Junctional complexes, mucus production and formation of glandular structures are generally accepted as signs of differentiation, while growth as single cells with cell contacts through desmosomes also are observed in undifferentiated cell cultures. In accordance with criteria of differentiation, growth kinetics in vitro were most vigorous in the least differentiated tumour, SK-ChA-1.

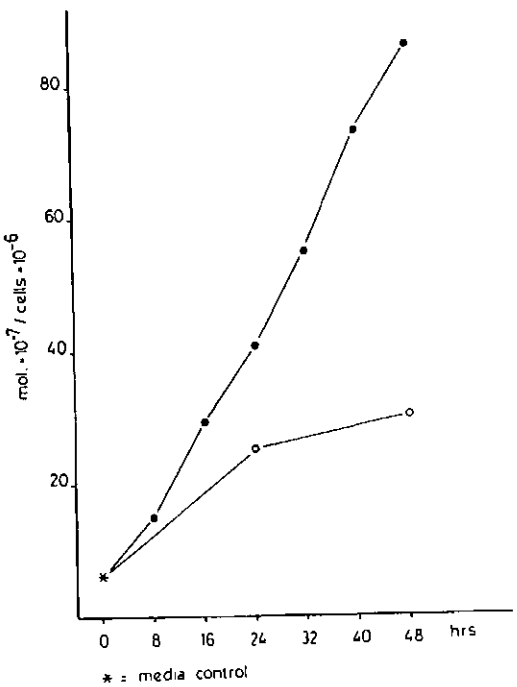


Fig. 7. Synthesis of C2 molecules as detected by a haemolytic assay in tissue culture supernatants, harvested every 8 (●) or 24 (○) hours up to 48 hours. The functional assay reflects the short half-life of biologically active C2 molecules.

After heterotransplantation to nude mice subcutaneous tumour growth was similar for Mz-ChA-1 and Mz-ChA-2 with doubling of tumour diameters approximately every 14 days, while SK-ChA-1 grew faster with tumour diameters doubling every 7–10 days. This correlates with growth kinetic data obtained from cell counts in tissue culture flasks, where SK-ChA-1 clearly was the fastest growing cell line. Cell doubling times of Mz-ChA-2 and SK-ChA-1 compare well with that reported for Colo 346 [6]. Cell kinetic data on other biliary adenocarcinoma cell lines have not been published [4,5].

The chromosomal complement in all three cell lines reported here allows for no clear distinction among them. SK-ChA-1 has occasionally a large dicentric marker chromosome and shows many structurally normal appearing trisomic elements. Modal number and distribution of chromosomes in these biliary adenocarcinoma cell lines compare well with data reported for other epithelial and mesothelial tumour cell lines [4].

Little is known about cell surface antigens on biliary tract cancers. Patterns of tumour-associated markers or differentiation antigens may aid histologic grading with respect to prognosis. It therefore seems worthwhile to dissect the antigenic phenotype of tumours with the help of monoclonal antibodies for use in immunohistology with the goal to define prognostic subgroups of tumour patients.

The cell surface antigen phenotype was determined with monoclonal antibodies proven to be of value in other human tumour systems [7–12]. For some antigenic systems, a differential pattern was defined which distinguished the three cell lines antigenically. Mz-ChA-1, the most differentiated cell line expressed gp 95, a transferrin-related molecule, and the M 19 antigen, a heat-labile structure not yet biochemically defined. Both are markers on malignant melanoma [7]. Only Mz-ChA-2, although intermediate within the morphological differentiation criteria, expressed an antigen (gp 130) which was not detected on the other two cell lines. All three cell lines expressed HLA-class I antigen but no HLA-class II antigens. Blood group A antigens were not expressed on any of the three cell lines. Mz-ChA-1 weakly expressed blood group B-like antigens. Both patients, Mz-ChA-1 and Mz-ChA-2, had an O,Rh phenotype in conventional blood group serology. The third patient's (SK-ChA-1) blood group is unknown. Blood group antigen expression in human epithelial cancers is well known but has not yet been reported for biliary adenocarcinomas [8,19–21]. Evidence is accumulating that normal and anomalous blood group antigens may be expressed on tumour cells, cross-reacting with conventional blood group antigens in routine assays with blood group typing reagents [19–21]. It is unclear to date whether normal or anomalous blood group antigen expression on malignant tumour cells may be considered to be differentiation markers or even are tumour antigens of autoimmunologic relevance. In this respect it may be of interest that natural antibodies to blood group antigens expressed on colon cancer cell cultures have been detected in healthy non-transfused individuals of a family at high risk for colorectal cancer [19].

The biochemical marker profile found in the cell lines described here shows a mutually exclusive tumour marker expression for either CEA or AFP. Mz-ChA-2, in addition to AFP, also has ferritin in spent tissue culture medium, the relevance of

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Yun-Yan Xiang

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Approved GMO		
Approved use of Animals	C57BL/6, BAL B/C Mice.	
Approved Toxin(s)		

] level 1 cell lines

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Brief Description of the use of Clone-9 and HepG2 cell lines

By Wei-yang Lu laboratory

Background information: Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the mammalian brain. GABA is synthesized from glutamate by the activity of glutamic acid decarboxylase (GAD). GABA mediates signaling through activation of A- and B- type GABA receptors (GABA-A and GABA-B receptors). It has been long known that GABA exists in the circulating blood (plasma). Recent studies, including ours, demonstrated that GAD and GABA receptors are also expressed in non-neuronal cells. However, the roles of GABA-signaling in non-neuronal cells are not clear. The circulating GABA in the blood increases when liver is injured; and the increased GABA has been proposed to be associated with hepatic encephalitis. Our recent studies revealed that GAD and GABA-A and GABA-B receptor might be expressed in hepatocytes (primary liver cells). We intend to study the roles and intracellular signaling of GABA in the regulation function of hepatocytes. To this end we need to use cell lines of hepatocytes.

Why we need the cell lines: Clone-9 is line of rat hepatocytes and **HepG2** is a line of human hepatocytes (both lines of cells are at level-1). We need the two lines of hepatocytes because most of experiments are done in the rat and to explore whether GABA signaling also exists in human hepatocytes we request the two hepatocyte lines.

What will do with the cells lines: We will culture the cells and use these cells for RT-PCR and immunocytochemistry assays of GAD and GABA receptors. Also, we will make patch-clamp recordings in the cells, examining whether activation of GABA receptor induces transmembrane current in the cells. These studies will provide us novel results of the GABA signaling in hepatocytes, which bear important clinical significance.

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Designations:	clone 9
Depositors:	ME Kaighn
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	Rattus norvegicus (rat)
Morphology:	epithelial
Source:	**Strain:** Sprague-Dawley **Organ:** liver **Disease:** normal
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Isolation:	**Isolation date:** 1968
Age:	4 weeks
Gender:	male
Comments:	Clone 9 (K-9) is an epithelial cell line isolated in 1968 from normal liver taken from a young male rat. The line has been used for studies of in vitro carcinogenesis and is useful clonal assays for screening sera and other nutritional supplements.
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Temperature:** 37.0°C
Subculturing:	**Protocol:**

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:6 is recommended
Medium Renewal: Every 2 to 3 days

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Preservation:	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2004 recommended serum:ATCC 30-2020
References:	21872: . Gene expression and carcinogenesis in cultured liver. New York: Academic Press; 1975. 22425: Weinstein IB, et al. Growth and structural properties of epithelial cell cultures established from normal rat liver and chemically induced hepatomas. Cancer Res. 35: 253-263, 1975. PubMed: 162864

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Shipped:	frozen
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Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial
	PHOTO
Source:	**Organ:** liver **Disease:** hepatocellular carcinoma
Cellular Products:	alpha-fetoprotein (alpha fetoprotein); albumin; alpha2 macroglobulin (alpha-2-macroglobulin); alpha1 antitrypsin (alpha-1-antitrypsin); transferrin; alpha1 antichymotrypsin; (alpha-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen; [3525] complement (C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (alpha-1 acid glycoprotein); alpha2 HS glycoprotein (alpha-2-HS-glycoprotein); beta lipoprotein (beta-lipoprotein); retinol binding protein (retinol-binding protein) [3525]
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Applications:	transfection host (*Nucleofection technology from Lonza* Roche FuGENE® Transfection Reagents)
Receptors:	insulin; insulin-like growth factor II (IGF II) [22446]
Tumorigenic:	No
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 10,11 D13S317: 9,13 D16S539: 12,13 D5S818: 11,12 D7S820: 10 F13A01: 5,7 F13B: 6,10 FESFPS: 11 LPL: 10,11 THO1: 9 TPOX: 8,9 vWA: 17
Cytogenetic Analysis:	modal number = 55 (range = 50 to 60); has a rearranged chromosome 1 [3525]
Age:	15 years adolescent
Gender:	male
Ethnicity:	Caucasian

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Comments: The cells express 3-hydroxy-3-methylglutaryl-CoA reductase and hepatic triglyceride lipase activities. [23557]
The cells demonstrate decreased expression of apoA-I mRNA and increased expression of catalase mRNA in response to gramoxone (oxidative stress). [26594]
There is no evidence of a Hepatitis B virus genome in this cell line. [1205] [22909]

Propagation: **ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.
Temperature: 37.0°C

Subculturing: **Protocol:**

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:6 is recommended
Medium Renewal: Twice per week

Preservation: **Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products: Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2003
recommended serum:ATCC 30-2020
derivative:ATCC CRL-10741
derivative:ATCC CRL-11997
purified DNA:ATCC *HB-8065D*

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Understand the role of chloride channel GABA-A-receptor in CF lung disease and its relationship to CFTR protein

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED. A GRANT SUMMARY PAGE MAYBE ADEQUATE IF IT PROVIDES SUFFICIENT DETAIL ABOUT EACH BIOHAZARD USED.

Names of all personnel working under Principal Investigators supervision in this location:

Yun-Yan Xiang
Shuangtian Wang

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ✓O YES O NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)?
If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? O YES ✓O NO
If YES, please give the name of the species. _____
What is the origin of the microorganism(s)? _____
Please describe the risk (if any) of escape and how this will be mitigated:

_____

_____

_____

Please attach the CFIA permit.
Please describe any CFIA permit conditions:

_____

_____

_____

1.2 Please complete the table below:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
A549	O Yes ✓O No	O Yes ✓O No	O Yes ✓O No	50 mL	ATCC	✓O1 O2 O3
BEAS-2B	O Yes ✓O No	O Yes ✓O No	O Yes ✓O No	50 mL	ATCC	O1 ✓O2 O3
IB3-1	O Yes ✓O No	O Yes ✓O No	O Yes ✓O No	50 mL	ATCC	O1 ✓O2 O3
IB3-B37	O Yes ✓O No	O Yes ✓O No	O Yes ✓O No	50 mL	ATCC	O1 ✓O2 O3

see table 2.3.9

*Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ✓O YES O NO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	O Yes ✓O No		Not applicable
Rodent	O Yes ✓O No		
Non-human primate	O Yes ✓O No		
Other (specify)	O Yes ✓O No		

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Supplier / Source
Human	☑ Yes ○ No	A549 IB3-1 BEAS-2B IB3-B37	ATCC
Rodent	○ Yes ☑ No		
Non-human primate	○ Yes ☑ No		
Other (specify)	○ Yes ☑ No		

*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ○ 1 ☑ 2 ○ 3

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ○ YES ☑ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Known to Be Infected With An Infectious Agent? YES/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		○ Yes ○ No		○ 1 ○ 2 ○ 3
Human Blood (fraction) or other Body Fluid		○ Yes ○ No		○ 1 ○ 2 ○ 3
Human Organs or Tissues (unpreserved)		○ Yes ○ No		○ 1 ○ 2 ○ 3
Human Organs or Tissues (preserved)		○ Yes ○ No		○ 1 ○ 2 ○ 3

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ○ YES ☑ NO If no, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ○ YES, complete table below ○ NO

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results

* Please attach a Material Data Sheet or equivalent if available.

4.3 Will genetic modification(s) involving viral vectors be made? O YES, complete table below Ø NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- HIV O YES, please specify _____ Ø NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens O YES, specify _____ Ø NO
- SV 40 Large T antigen Ø YES BEAS-2B O NO
- E1A oncogene O YES IB3-1 Ø NO
- Known oncogenes O YES, please specify _____ Ø NO
- Other human or animal pathogen and or their toxins O YES, please specify _____ Ø NO

4.5 Will virus be replication defective? O YES Ø NO

4.6 Will virus be infectious to humans or animals? O YES Ø NO

4.7 Will this be expected to increase the containment level required? O YES Ø NO

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted involving a biological agent? O YES Ø NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

5.2 If YES, please specify which biological agent will be used: _____
Please attach a full description of the biological agent.

5.2 Will the biological agent be able to replicate in the host? O YES O NO

5.3 How will the biological agent be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted:_____

5.5 Has human ethics approval been obtained? O YES, number: _____ O NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? Ø YES O NO If no, please proceed to section 7.0

6.2 Name of animal species to be used C57BL/6, BALB/c mice

6.3 AUS protocol # to be submitted

6.4 Will any of the agents listed be used in live animals O YES, specify: _____ Ø NO

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other body fluids including blood be used?

- Pound source dogs O YES √Ø NO
- Pound source cats O YES √Ø NO
- Cattle, sheep or goats O YES √Ø NO
- Non-human primates O YES, please specify species _____ √Ø NO
- Wild caught animals O YES, please specify species & colony # _____ √Ø NO
- Birds O YES √Ø NO
- Others (wild or domestic) O YES, please specify _____ √Ø NO

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? O YES √Ø NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s)_____
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin_____

8.4 How much of the toxin is handled at one time*? _____

8.5 How much of the toxin is stored*? _____

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

9.0 Insects Requiring CFIA Permits

9.1 Do you use insects that require a permit from the CFIA? O YES √Ø NO
If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. _____

9.3 What is the origin of the insect? _____

9.4 What is the life stage of the insect? _____

9.5 What is your intention? O Initiate and maintain colony, give location: _____
O "One-time" use, give location: _____

9.6 Please describe the risk (if any) of escape and how this will be mitigated:
_____
_____
_____

9.7 Please attach the CFIA permit.

9.8 Please describe any CFIA permit conditions:
_____
_____
_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

10.0 Plants Requiring CFIA Permits

10.1 Do you use plants that require a permit from the CFIA? O YES ✓Ø NO
If no, please proceed to Section 11.0

10.2 If YES, please give the name of the species. _____

10.3 What is the origin of the plant? _____

10.4 What is the form of the plant (seed, seedling, plant, tree...)? _____

10.5 What is your intention? O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO
If yes, please describe: _____
_____

10.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:
_____
_____
_____

10.8 Is the CFIA permit attached? O YES O NO
If NO, please forward the permit to he Biosafety Officer when available.

10.9 Please describe any CFIA permit conditions:
_____
_____
_____

11.0 Import Requirements

11.1 Will any of the above agents be imported? O YES, please give country of origin_____
If no, please proceed to Section 12.0 ✓Ø NO

11.2 Has an Import Permit been obtained from HC for human pathogens? O YES O NO

11.3 Has an import permit been obtained from CFIA for animal or plant pathogens? O YES O NO

11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ O NO

12.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

SIGNATURE Wenjiang Liu

13.0 Containment Levels

11.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. O 1 Ø 2 O 3

13.2 Has the facility been certified by OHS for this level of containment?

O YES, permit # if on-campus_____

Ø NO, please certify

O NOT REQUIRED for Level 1 containment

→ Level 2 Inspection completed Nov 17/09 SL.

14.0 Procedures to be Followed

14.1 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca/

SIGNATURE Wenjiang Liu Date: Sept. 16, 2009

15.0 Approvals

UWO Biohazard Subcommittee: SIGNATURE: S.M. Kelly
Date: 18 Nov. 2009

Safety Officer for Institution where experiments will take place: SIGNATURE: Ronald Moseley
pending Level 2 Inspection Date: Sept. 22, 2009

Safety Officer for University of Western Ontario (if different from above): SIGNATURE: JStanley
Date: Nov. 17/09

Approval Number: BIO-RA-0056 Expiry Date (3 years from Approval): Nov 17, 2012

Special Conditions of Approval:

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

Funding Agency: Canadian Institutes of Health Research

Grant Title: **GABAergic regulations of airway epithelium in asthma**

During the development of asthma, airways in the lung undergo structural remodeling and functional alterations, which are characterized by goblet cell (GC) hyperplasia and airway hyper-responsiveness (AHR). The mechanism of such asthmatic reactions remains unclear. Novel data from our recent studies have shown that airway epithelial cells (AECs) in the lung express glutamic acid decarboxylase (GAD), the key enzyme for the synthesis of γ-aminobutyric acid (GABA), the major inhibitory transmitter in the brain. Furthermore, AECs also express subunits for A-type GABA receptors ($GABA_AR$s), and these subunits are known to form chloride channels in neurons. Our preliminary results strongly suggest that $GABA_AR$s provide an unexpected excitatory and autocine/paracrine function in AECs. Remarkably, the levels of GAD and $GABA_AR$ subunits expressed in AECs increased dramatically in BALB/c mice that were sensitized and challenged with ovalbumin (OVA), a widely used animal model of asthma. The present project intends to study the role of GABA and $GABA_AR$s in asthmatic reaction, with particular regard to the underlying mechanisms for AEC transdifferentiation.

To demonstrate the role of the AEC GABAergic system in asthmatic reactions, the allergen-sensitive BLAB/c mice and allergen-insensitive C57BL/6 mice will be sensitized with OVA, and then challenged with OVA. The expression levels of GAD and $GABA_AR$s in AECs of allergen-challenged mice will be examined and correlated to the changes of airway resistance. Allergen-challenged BALB/c mice will be treated intranasally with $GABA_AR$ inhibitor; to examine whether blocking GABA signaling improves pulmonary functions.

Our preliminary data showed that T_{H2} cytokine interleukine-13 (IL-13) increased in the lung of OVA-treated BALB/c mice. Intranasal administration (i.n.) of IL-13 enhanced GAD and $GABA_AR$s in AECs. Conversely, i.n. $GABA_AR$ inhibitor suppressed the extent of CG hyperplasia and mucus production, but did not affect the level of IL-13 in the lung of the OVA-challenged mice. These results suggest that in asthma the GABAergic activation in AECs is downstream of the IL-13-initiated signaling. In neurons, phosphorylation of the $GABA_AR$ β_2-subunit by Akt initiates translocation of the receptor to the plasma membrane. In addition, $GABA_AR$ activation depolarizes the neural progenitors, consequently triggering neural proliferation and differentiation. To explore the pathway by which T_{H2} cytokines enhances $GABA_AR$ expression in AECs, we will determine whether similar mechanisms exist in the AECs. IL-13 will be applied to BALB/c mice to determine whether it activates PI3K/Akt and phosphorylates $GABA_AR$ in AECs. PI3K or Akt inhibitor will be administrated before application of cytokines to determine whether PI3K/Akt signaling regulates the T_{H2} cytokine-increased expression of $GABA_AR$s in AECs. AECs, namely A549 and BEAS-2B cells, grown in air-liquid surface will be treated with IL-13 to show that IL-13 increases $GABA_AR$s in the apical membrane of AECs.

We hypothesize that, during the development of asthma, T_H2 cytokines, including Il-13, enhance the expression of GAD and $GABA_AR$s in AECs. Consequently, the activated-GABAergic signaling induces AEC transdifferentiation, and hence alterations of airway function. This study may lead to novel treatments of asthma.

Funding Agency: Canadian Cystic Fibrosis Foundation

Grant Title: **Understand the role of chloride channel GABA-A-receptor in CF lung disease and its relationship to CFTR protein**

Cystic fibrosis (CF), a common genetic disorder, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) which is a membrane chloride channel located at airway epithelial cells. There are other chloride channels existing in airway epithelial cells and may be able to reverse the effects of CF. Recent studies in our laboratory indicated that a specific chloride channel – GABA-A-receptor is located on the epical membrane of airway cells and an autocrine/paracrine GABA signaling system do existent in bronchial epithelial cells. Deficit in CFTR function increase the expression of GABA-signaling molecules. In this study, GABA-signaling in bronchial epithelial cells (BECs) will be investigated both in $CFTR^{-/-}$ mice and matched wild-type C57BL/6 mice, and in several cultured lines of human BECs, namely BEAS-2B, IB3-1 (BEC line isolated from a patient with cystic fibrosis) and IB3-837 (IB3-1 expressing wild type CFTR, a cell line from other research laboratory at the University of Toronto) cells by Western blot, immunohistochemistry/ immunocytochemistry and patch-clamp recordings. This proposal initiates studies of the interactions between CFTR and GABA signaling in the cells. Understanding the crucial role of GABA signaling in airway epithelial cells in the pathological course of CF lungs will provide a new target for therapies of CF.

Cell Biology

ATCC® Number:	CCL-185™	Order this Item	Price:	$256.00

Designations:	A549
Depositors:	M Lieber
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial 
Source:	**Organ:** lung **Disease:** carcinoma
Cellular Products:	keratin
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Isolation:	**Isolation date:** 1972
Applications:	transfection host (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents)
Reverse Transcript:	negative
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 10,12 D13S317: 11 D16S539: 11,12 D5S818: 11 D7S820: 8,11 THO1: 8,9.3 TPOX: 8,11 vWA: 14
Cytogenetic Analysis:	This is a hypotriploid human cell line with the modal chromosome number of 66, occurring in 24% of cells. Cells with 64 (22%), 65, and 67 chromosome counts also occurred at relatively high frequencies; the rate with higher ploidies was low at 0.4%. There were 6 markers present in single copies in all cells. They include der(6)t(1;6) (q11;q27); ?del(6) (p23); del (11) (q21), del(2) (q11), M4 and M5. Most cells had two X and two Y chromosomes. However, one or both Y chromosomes were lost in 40% of 50 cells analyzed. Chromosomes N2 and N6 had single copies per cell; and N12 and N17 usually had 4 copies.
Isoenzymes:	G6PD, B

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Age:	58 years
Gender:	male
Ethnicity:	Caucasian
Comments:	This line was initiated in 1972 by D.J. Giard, et al. through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. [23218] Further studies by M. Lieber, et al. revealed that A549 cells could synthesize lecithin with a high percentage of desaturated fatty acids utilizing the cytidine diphosphocholine pathway. [58030] The cells are positive for keratin by immunoperoxidase staining.
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C
Subculturing:	**Protocol:** 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. Cultures can be established between 2 X 10(3) and 1 X 10(4) viable cells/cm2. Do not exceed 7 X 10(4) cels/cm2. 6. Incubate cultures at 37°C. **Interval:** Maintain cultures at a cell concentration between 6 X 10(3) and 6 X 10(4) cell/cm2. **Subcultivation Ratio:** A subcultivation ratio of 1:3 to 1:8 is recommended **Medium Renewal:** 2 to 3 times per week
Preservation:	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Doubling Time:	about 22 hours

Related Products:

recommended serum:ATCC 30-2020
Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2004

References:

23218: Giard DJ, et al. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer Inst. 51: 1417-1423, 1973. PubMed: 4357758
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32373: Goodrum FD, et al. Adenovirus early region 4 34-kilodalton protein directs the nuclear localization of the early region 1B 55-kilodalton protein in primate cells. J. Virol. 70: 6323-6335, 1996. PubMed: 8709260
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32514: Bartz SR, et al. Human immunodeficiency virus type 1 cell cycle control: Vpr is cytostatic and mediates G2 accumulation by a mechanism which differs from DNA damage checkpoint control. J. Virol. 70: 2324-2331, 1996. PubMed: 8642659
32722: Garofalo R, et al. Transcriptional activation of the interleukin-8 gene by respiratory syncytial virus infection in alveolar epithelial cells: nuclear translocation of the RelA

transcription factor as a mechanism producing airway mucosal inflammation. J. Virol. 70: 8773-8781, 1996. PubMed: 8971006
32758: Jamaluddin M, et al. Inducible translational regulation of the NF-IL6 transcription factor by respiratory syncytial virus infection in pulmonary epithelial cells. J. Virol. 70: 1554-1563, 1996. PubMed: 8627674
33091: Lewis JA, et al. Inhibition of mitochondrial function by interferon. J. Biol. Chem. 271: 13184-13190, 1996. PubMed: 8662694
58030: Lieber M, et al. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int. J. Cancer 17: 62-70, 1976. PubMed: 175022

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Cell Biology

ATCC® Number: CRL-9609™ Order this Item **Price:** $256.00

Designations: BEAS-2B

Depositors: The United States of America

Biosafety Level: 2 [CELLS CONTAIN PAPOVAVIRUS]

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: *Homo sapiens* (human)

Morphology: epithelial

Source:
Organ: lung
Tissue: bronchus
Disease: normal
Cell Type: epithelialvirus transformed

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Applications: transfection host (Roche FuGENE® Transfection Reagents)

Tumorigenic: No

Comments:
Epithelial cells were isolated from normal human bronchial epithelium obtained from autopsy of non-cancerous individuals. [21937]
The cells were infected with an adenovirus 12-SV40 virus hybrid (Ad12SV40) and cloned. [21937]
The cells retain the ability to undergo squamous differentiation in response to serum, and can be used to screen chemical and biological agents for ability to induce or affect differentiation and/or carcinogenesis. [21937]
The cells stain positively for keratins and SV40 T antigen.

Propagation: **ATCC complete growth medium:** The base medium for this cell line (BEBM) along with all the additives can be obtained from Lonza/Clonetics Corporation as a kit: BEGM, Kit Catalog No. CC-3170. ATCC does not use

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the GA-1000 (gentamycin-amphotericin B mix) provided with the BEGM kit. Note: Do not filter complete medium.
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C
Growth Conditions: The flasks used should be precoated with with a mixture of 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I and 0.01 mg/ml bovine serum albumin dissolved in BEBM medium .

Subculturing:

Protocol:

1. Remove and discard culture medium.
2. Add 2.0 to 3.0 ml of 0.25% Trypsin - 0.53mM EDTA solution containing 0.5% polyvinylpyrrolidone (PVP) to flask and observe cells under an inverted microscope until cell layer is dispersed (usually with 5 to 10 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37C to facilitate dispersal.
3. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
4. Transfer cell suspension to centrifuge tube and spin at approximately 125 x g for 5 to 10 minutes.
5. Discard supernatant and resuspend cells in fresh growth medium. Inoculate new flasks at 1500 to 3000 cells per sq. cm. The culture flasks used should be pre-coated with a mixture of 0.01mg/ml fibronectin, 0.03 mg/ml bovine collagen type I and 0.01mg/ml bovine serum albumin dissolved in BEBM medium (see reference below).
6. Place culture flasks in incubators at 37C.

Interval: Subcultured before reaching confluence.
Medium Renewal: Every 2 to 3 days

Preservation:

Freeze medium: Complete growth medium supplemented with 1% PVP and 7.5% DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

0.25% (w/v) Trypsin - 0.53 mM EDTA in Hank' BSS (w/o Ca++, Mg++):ATCC 30-2101
Cell culture tested DMSO:ATCC 4-X

References:

21937: Reddel RR, et al. Immortalized human bronchial epitherial mesothelial cell lines. US Patent 4,885,238 dated Dec 5 1989
22301: Lechner JF, LaVeck MA. A serum-free method for culturing normal human bronchial epithelial cells at clonal density. J. Tissue Culture Methods 9: 43-48, 1985.
30067: Sakamoto O, et al. Role of macrophage-stimulating protein and its receptor, RON tyrosine kinase, in ciliary motility. J. Clin. Invest. 99: 701-709, 1997. PubMed: 9045873

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Designations: IB3-1 [JHU-S2]

Depositors: PL Zeitlin

Biosafety Level: 2 [Cells contain SV40 and Adenovirus 12 DNA viral sequences]

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: *Homo sapiens* (human)

Morphology: epithelial

PHOTO

Source:
Organ: bronchus
Disease: cystic fibrosis
Cell Type: epithelial immortalized with Ad12-SV40 hybridimmortalized with adenovirus 12 - SV40 virus hybrid (Ad12-SV40)

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Restrictions: Part of the Johns Hopkins Special Collection

Isolation: **Isolation date:** 1992

Cytogenetic Analysis: modal chromosome number = 80 to 90.
There are an average of four chromosome 7 per cell. The phenylalanine 508 deletion in the gene coding for the cystic fibrosis transmembrane regulator is present on at least one chromosome. [70685]

Age: 7 years

Gender: male

Ethnicity: White

Comments: IB3-1 (ATCC CRL-2777) is an immortalized cell line created in 1992 from a primary culture of

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bronchial epithelia cells isolated from a patient with cystic fibrosis. The culture was transformed with a hybrid virus, adeno-12-SV40 [PubMed: 1849726]. The IB3-1 are deficient in cyclic AMP-medicated protein kinase A activation of chloride conductance, which is diagnostic of Cystic Fibrosis [PubMed: 7679117]. Genotypically, the cell line is a compound heterozygote containing the delta F508 mutation and a nonsense mutation, W1282X, with a premature termination signal [PubMed: 10518596].The cells stain positively for SV40 T antigen [PubMed: 1849726].They can be used for studies of the mutant cystic fibrosis transmembrane regulatory protein and its interaction with the chloride channel.The S9 cell line (ATCC CRL-2778) and the C38 cell line (ATCC CRL-2779) were derived from the IB3-1 cell line.The CF phenotype present in the IB3-1 cells was corrected in the S9 and C38 cell line by transfection with wild-type adeno-associated viral cystic fibrosis transmembrane conductance regulator (AAVCFTR).

Propagation:

ATCC complete growth medium: LHC-8 Basal Medium (Invitrogen catalog #12679-015), 95%; fetal bovine serum, 5%
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C
Growth Conditions: The flasks used should be precoated with a mixture of 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I and 0.01 mg/ml bovine serum albumin dissolved in culture medium.

Subculturing:

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37° C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new precoated culture vessels.
   An inoculum of 3 X 10(3) to 8 X 10(3) viable cells/cm2 is recommended. Do not exceed 1 X 10(5) cells/cm2..
6. Incubate cultures at 37°C.

Interval: Maintain cultures at a cell concentration between 4 X 10(3) and 4 X 10(4) cells/cm2.
Subcultivation Ratio: A subcultivation ratio of 1:6 to 1:10 is recommended
Medium Renewal: Two to three times weekly

Preservation:

Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Doubling Time:

29 hrs

Related Products:

source culture:ATCC JHU-52
derivative:ATCC CRL-2778
recommended serum:ATCC 30-2020
derivative:ATCC CRL-2779

References:

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