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SOME PROPERTIES
OF THE *EPITHELIOMA PAPULOSUM CYPRINI* (EPC)
CELL LINE FROM CARP *CYPRINUS CARPIO*

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SUMMARY

A cell line, named *Epithelioma papulosum cyprini* (EPC) and originating from carp epidermal herpes virus-induced hyperplastic lesions, was established and has now given rise to more than 80 subcultures. It grows within a wide temperature range (15-33° C), survives between 7 and 10° C for several months without alterations in its further virus susceptibility and growth characteristics, and degenerates at 37° C. Its karyotype is $2n = 96$. It propagates most of the systemic infection viruses of the fish families *Salmonidae*, *Cyprinidae*, *Anquillidae* and *Esocidae*, as well as Indiana-type vesicular stomatitis virus.

Despite the origin of EPC cells, we were unable to demonstrate the presence of herpes virus in them. Their transformation was reflected in their karyotype (normally $2n = 100$) and by their growth ability in soft agar medium; monolayer growth, however, was inhibited at a density of 3×10^6 cells/cm².

The temperature growth range, good splitting ratio (1/10) and virus susceptibility make EPC cells a highly suitable material both for fish pathology and for comparative virology studies.

KEY-WORDS: *Epithelioma papulosum cyprini*, Carp pox, Herpes virus, Cell culture; Karyotype, Rhabdoviruses, Fish pathology.

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INTRODUCTION

During experiments on the etiology of carp pox carried out in 1969 in Yugoslavia by two of us (N. F. and D. S.), a cell line was initiated and called *Epithelioma papulosum cyprini* (EPC) because of the origin of the cells.

Despite our failure to cultivate the so-called carp pox virus, EPC culture procedures were continued because of the potential interest of obtaining a cell line as a useful tool in diagnosis and research on viral diseases of the carp, one of the most widespread freshwater fish species in the world.

This paper describes the establishment of the EPC cell line, which has now been maintained for 12 years over 80 subcultures, and discusses its growth properties and virus susceptibility.

MATERIALS AND METHODS

Method of establishment.

Two carp weighing about 750 g each, which originated from a fish farm and exhibited proliferative skin lesions typical of carp pox, were kept for 10 days in an aquarium supplied with free-flowing aerated water, chlorine content 0.2 mg/l.

Several epitheliomatous outgrowths of the skin were sampled with a scalpel and minced in a beaker with scissors. Tissue fragments were washed three times in Eagle's minimal essential medium (MEM) supplemented with 2,000 i. u. of penicillin/ml and 2,000 µg of streptomycin/ml, and subsequently kept in that medium for 1 h at 22° C. After washing in pH 7.2 phosphate-buffered saline (PBS), minced skin samples were treated overnight at 4° C by trypsin 0.25 % in PBS [23]. Harvested cells were centrifuged in the cold at 300 g for 30 min, then washed once in PBS and finally suspended in growth medium consisting of MEM supplemented with 10 % foetal bovine serum plus 200 i. u. of penicillin and 200 µg of streptomycin/ml. Buffer was sodium bicarbonate (pH 7.2-7.6). Cell suspensions (10⁶ cells/ml) were distributed in glass tubes (2 ml/tube) and incubated at 22° C.

Subcultivation of the primary culture was achieved by EDTA-trypsin solution [19], and subcultures were grown either in MEM as previously described, or, after the 52nd subculture, in Stoker's medium [18] (Eurotroph Eurobio) buffered by tris-HCl 0.16 M at pH 7.4, and supplemented with 10 % foetal bovine serum, 10 % tryptose phosphate broth and antibiotics, as above.

Virological checking of the cells.

Because herpes virus had been demonstrated in skin proliferations of the so-called carp pox [17], attempts were made to assess the presence of such a virus in EPC cells.

CPE = cell-plating efficiency.
 EM = electron microscope.
 FHM = fathead minnow.
 IHN = infectious haematopoietic necrosis.
 IPN = infectious pancreatic necrosis.
 i. u. = international unit.
 MEM = minimal essential medium.

NDV = Newcastle disease virus.
 PBS = phosphate-buffered saline.
 PFR = pike fry rhabdovirus.
 pfu = plaque-forming unit.
 SVC = spring viremia of carp.
 VSV = vesicular stomatitis virus.

First, the culture medium of subcultures 3 and 10 was inoculated onto primary cell cultures of carp ovary [11]. Medium from subculture 15 was then inoculated into fathead minnow (FHM) cells [5], and two subsequent blind passages were performed.

Next, ultrathin sections of different subcultures were examined in electron microscopy both in Switzerland (by one of us: L. O. Z.) and in France (by S. C.). The first cell sample was taken after 15 subcultures and fixed *in situ* by 4 % formaline in Millonig's phosphate buffer for 30 min, then scraped off with a rubber policeman pelleted by centrifugation, post-fixed in 2 % osmium tetroxide, dehydrated in ethanol, prestained in 1 % uranyl acetate, washed in ethanol and embedded in epoxy-eosin « Dureopan ACM ». Samples were sectioned with an « LKB-III » ultratome and the ultrathin sections were post-stained with lead citrate and examined in a « Philips 200 » electron microscope (EM) under 80 kV.

The second examination involved cell cultures sampled between subcultures 62 and 72 fixed in 1.6 % glutaraldehyde in Sorensen's buffer (pH 7.2) for 30 min at 4° C and subsequently processed as previously described, except that they were embedded in Epon, sectioned with « LKB-III » ultratome and observed in a « Philips 300 » EM.

Recently, EPC cells used in several American laboratories have been reported to be chronically infected with a syncytia-inducing coronavirus [9]. Since such a condition appeared only at incubation temperatures above 30° C, ten successive subcultures were carried out at one-week intervals. Each time, samples of cells grown on glass coverslips were collected, Giemsa-stained and microscopically examined for syncytia. Coverslips from the final subculture were also tested by indirect immunofluorescence [19] for presumptive infection by a coronavirus belonging either to OC49 or to the 229E group [22]. Parallel to this, coverslip cultures from cells which had not undergone previous storage at high temperature were infected with a bovine enteric coronavirus strain (OC43 group) at multiplicities of infection of 2 and 10 pfu/cell, respectively, then incubated at 35° C and further examined either for syncytia or for immunofluorescence plaques.

Chromosomal analysis.

One-day old cells of subculture 65 were treated with Colcemid (0.02 µg/ml), then dislodged with versene, treated hypotonically with PBS diluted 1/5 in distilled water and fixed with methanol-glacial acetic acid 3/1 before Giemsa-staining and karyological analysis [7].

Growth curves.

Cell growth at various temperatures was studied in duplicate 25-cm² flasks (Corning) seeded with 8×10^6 cells in 6 ml of Stoker's medium, which were first held at 30° C for 2 h to allow them to attach, and then further incubated at 7, 10, 15, 20, 30, 33 and 37° C. Two flasks of each series were sampled at various intervals, and the number of viable cells was determined using the trypan blue exclusion technique and haemocytometer counts.

Cell growth as a function of the initial cell number was studied by seeding 1 ml of a 2-fold dilution of a cell suspension primarily containing 1.6×10^7 cells/ml into 25-cm² flasks, each containing 5 ml of Stoker's medium. Five series of flasks containing 1.6×10^7 , 8×10^6 , 4×10^6 , 1×10^6 and 0.25×10^6 cells, respectively, were incubated at 30° and cells counts in duplicate flasks were performed at various dates between days 2 and 21 post-seeding.

All growth experiments were performed with subcultures 62 to 65.

Cell storage.

Cell preservation was achieved by freezing at -196° C. For that purpose, EPC cells undergoing the logarithmic phase of their growth curve were harvested,

centrifuged at 300 *g* for 5 min and resuspended at a cell density of 25 million cells/ml in Stoker's medium containing 10 % foetal bovine serum and 10 % tryptose phosphate broth supplemented with 10 % dimethyl sulphoxide (v/v). The cell suspension was distributed per millilitre into plastic tubes designed for nitrogen freezing (Nunc). Gradual lowering of the temperature (0.5-1° C per min) was achieved by placing the tubes into cylindrical channels bored in a polystyrene block stored in a freezer (-70° C). Three h later, the frozen tubes were transferred into a nitrogen tank.

Plating efficiency and cloning.

A suspension of EPC cells was obtained from a monolayer of EPC (62nd sub-culture) and serially diluted 10-fold until suspensions of approximately 1,000, 100 and 10 cells/ml were obtained. One-ml volumes of each suspension were distributed into 6 cm diameter Petri dishes (Falcon), and 3 ml of Stoker's medium were added to the dishes, which were incubated at 30° C without being moved, except for one which was checked every two days for cell attachment. After 16 days, the cell culture medium was withdrawn and the remaining microcolonies were rinsed, then fixed for 2 h in 10 % formalin, stained with 1 % crystal violet and enumerated. A microcolony from an unfixed dish was sampled in 150 µl of 0.04 % versene in PBS, transferred to a plastic cell-culture tube (Nunc) and subsequently sampled 7 times in 25-cm² and 75-cm² flasks before being checked for plating efficiency, virus propagation and freezing.

Growth in semi-solid suspension culture.

The degree of autonomy acquired by EPC cells had previously been tested by the soft agar culture technique [13]. Namely, EPC cells were suspended in 0.5 % agar (Difco Bacto-agar) Stoker's complete medium kept at 44° (one volume of cells per two volumes of medium); 2 ml of this suspension was layered onto the surface of solidified 0.5 % agar Stoker's medium in 25-cm² duplicate plastic culture flasks (Corning). Cell number had previously been adjusted to obtain cell densities of 10³ and 10⁴ cells per ml, respectively, with both uncloned and cloned EPC cells. Parallel series of flasks received the same amount of cells in agar medium supplemented with DEAE-dextran (50 µg/ml) [13].

Virus propagation.

EPC cell susceptibility to fish viruses was tested with the following virus strains previously grown either in FHM cells or in RTG-2 cells [24], or directly extracted from diseased fish: Egtved virus strains of several serotypes [21, 12], infectious haematopoietic necrosis (IHN) virus [1], spring viremia of carp (SVC) virus [5], pike fry rhabdovirus (PFR) [10], eel rhabdoviruses [16, 2], infectious pancreatic necrosis virus strains of the serotype American-type culture collection VR 299 and serotypes Sp or Ab [20].

Three virus strains of warm-blooded vertebrates, namely, vesicular stomatitis virus (VSV) Indiana grown in BHK cells, Newcastle disease virus (NDV) grown in chick embryo cells and Reovirus 3 grown in L cells were also inoculated into 1-day old EPC cells. The virus yields were titrated by plaque assay either under a 0.4 % agarose overlay for fish viruses and VSV, or in liquid medium for NDV and Reovirus.

All viruses tested were allowed to adsorb on the cells for 1 h at the usual incubation temperature (table II); inocula were then withdrawn and the cell sheet rinsed 3 times with Stoker's medium before adding the final medium containing 2 % foetal bovine serum.

RESULTS

Establishment of EPC cell line.

In 30 primary test-tube cultures, cells adhered well, but during the first 7-10 days most degenerated and became necrotic. Occasional small groups of replicating fibroblast-like cells appeared in a few test tubes between day 6 and day 20. After 60 days, a confluent sheet of predominantly fibroblast-like cells was formed in one tube, which was subcultivated. The first four splittings of monolayers were performed at ratios of 1/1 at 20-22° C. Thereafter, the splitting ratio was 1/2 to 1/4.

During the first 13 passages, EPC cells were predominantly fibroblast-like. After the 13th passage, epithelial-like cells predominated. Such cells were cultivated both in Yugoslavia and in France. The morphology of the Zagreb strain began to change to a fibroblast-like morphology from the 41st passage on but this strain was lost. All other strains of EPC cells had an epithelial-like morphology (fig. 1).

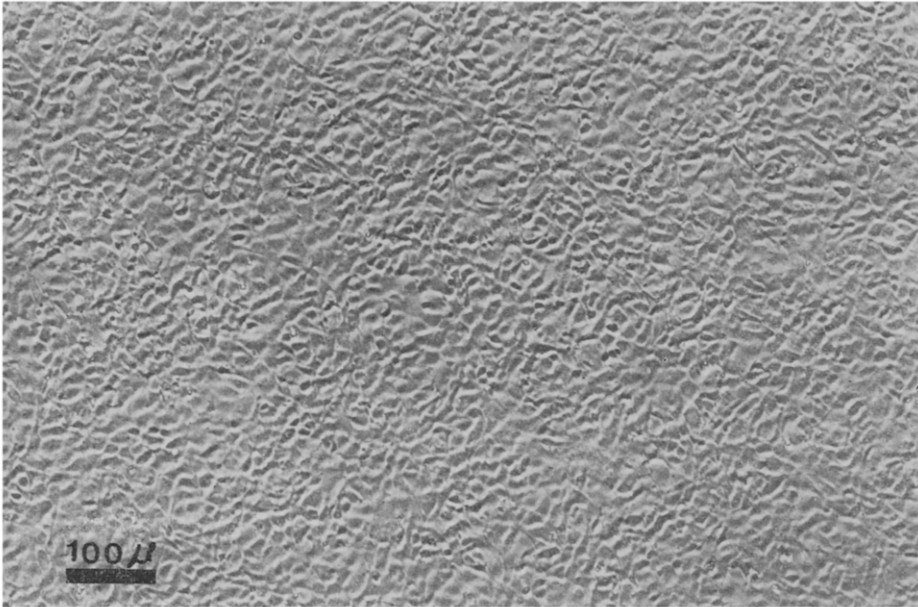


FIG. 1. — Aspect of a 2-day old culture in a 75-cm² plastic flask seeded with 3.2×10^5 cells/cm² (phase contrast objective 10 x).

Virological checking of the EPC-cell line.

Neither primary cultures of carp ovary nor FHM cells inoculated with the medium nor cell extracts from an early or late subculture of EPC developed any cytopathic effect, even through further blind passages.

All of these assays were performed both at 15 and at 30° C. In addition, viral particles were never found in the ultra-thin sections of EPC cells examined by EM.

Likewise, all examinations undertaken to reveal a coronavirus infection remained unsuccessful. No syncytia appeared, and no fluorescent plaques were observed following several subcultures at 33° C. Finally, none of the attempts to provoke infection by the OC49 virus strain resulted in fluorescent plaques.

Chromosome number.

Approximately 21 % of the 137 metaphases from the 65th EPC subculture (table I) had the modal number of 96 chromosomes. Most of the other metaphases (48 %) had between 94 and 99 chromosomes. Chromosome frequencies between 84 and 93 were found in 18 % and those between 100 and 107 in 13 % of metaphases.

TABLE I. — Distribution of the chromosome number following enumeration in 137 cells.

Chromosome number	84-90	91	92	93	94	95	96	97	98	99	100	101	102-107
Number of cells	6	4	8	7	17	11	29	14	12	12	6	4	7

Growth curves.

As soon as cells attached to the flask surface, they exhibited a loose confluency.

The results of the experiments on cell growth as a function of the incubation temperature are reported in figure 2. EPC cells were viable from 7.5° C up to 33° and were able to grow between 15 and 33° C. The optimal growth temperature was 30° C, at which temperature cells underwent one division at approximate 2-day intervals until they reached a tight confluency leading to density-dependent growth inhibition D. When seeded with 8 million cells/25-cm² flask, *i. e.* 3.2×10^5 cells/cm², this occurred within 6-7 days, at which time the cell number/cm² ranged between 2.6 and 3 million cells with each cell dividing about 3 times. Once the plateau was reached, some cells began to degenerate, rather slowly during the first month and more rapidly thereafter. At 33° C, the decrease in viable cell number became severe after 3 weeks, with 15.4 million remaining on day 28 and none remaining on day 60. At 20° C, the kinetics of growth was about half that at 30° C, which is consistent with such biological mechanisms; the final cell harvest, however, was unmodified, and the same final result was obtained at 15° C. At 7 and 10° C, EPC cells survived but did not divide. They remained perfectly viable, and when transferred to 30° C on day 60, underwent the same growth as previously reported at this temperature, exhibiting the same susceptibility to viruses as that of

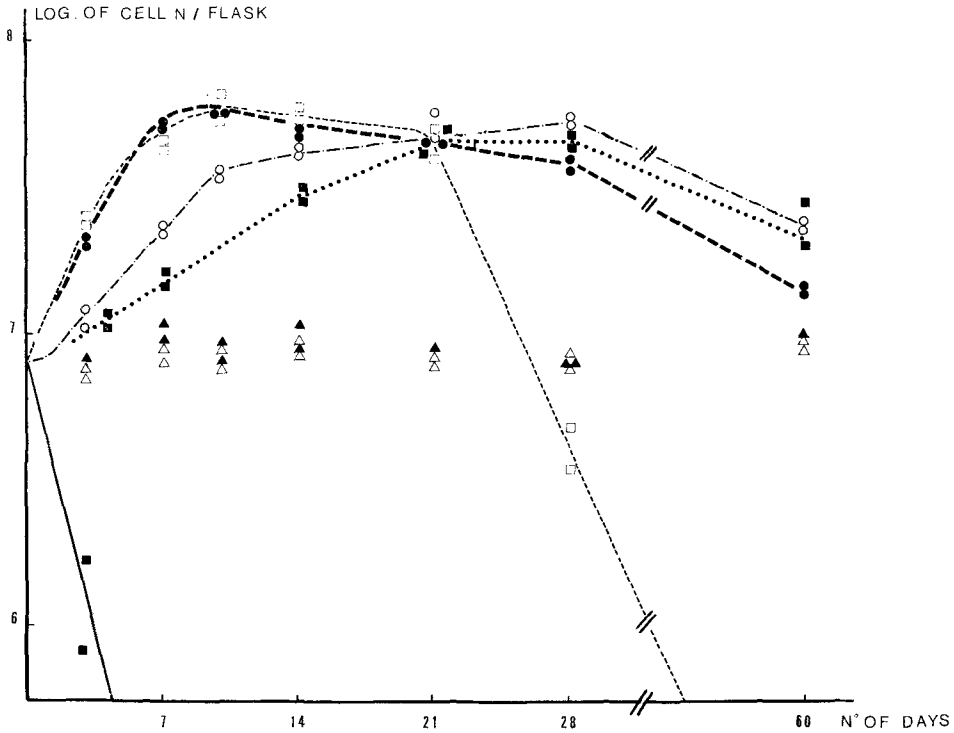


FIG. 2. — EPC-cell growth as a function of the incubation temperature.

Culture flasks (Corning 25 cm²) containing 6 ml of Stoker's medium were seeded with 8 million cells and divided into 6 groups, each incubated at one of the following temperatures: 7° C = Δ; 10° C = ▲; 15° C = ■●●■; 20° C = ○; 30° C = ●; 33° C = □; 37° C = ■—■. Duplicate monolayer cultures were split at weekly intervals during the first month and at the end of the second. Results are expressed as the number of viable cells per flask, with percent of viability of each harvest estimated by the trypan blue exclusion method.

freshly subcultivated cells. On the contrary, EPC cells did not survive at 37° C and no viable cells were found after 10 days.

Growth occurring in flasks seeded at various cell densities (fig. 3) and incubated at 30° C always led to the same final value, *i. e.* 2.6 to 3 × 10⁶ cells/cm². This saturation density was reached within 14 days in flasks seeded with 4 × 10⁶ cells, and in 24-26 days in flasks seeded with 1 × 10⁶ and 0.25 × 10⁶ cells, respectively. With the two highest inocula, saturation density was reached within less than 8 days, which is consistent with results reported in figure 2.

Cell preservation.

The recovery of viable cells was higher than 98 % following rapid thawing at 37° C, seeding into Stoker's medium and incubation at 30° C. The quantity of cells contained in a frozen tube, when seeded into a 75-cm² flask, led to a cell harvest of 200-250 million cells within 7 days.

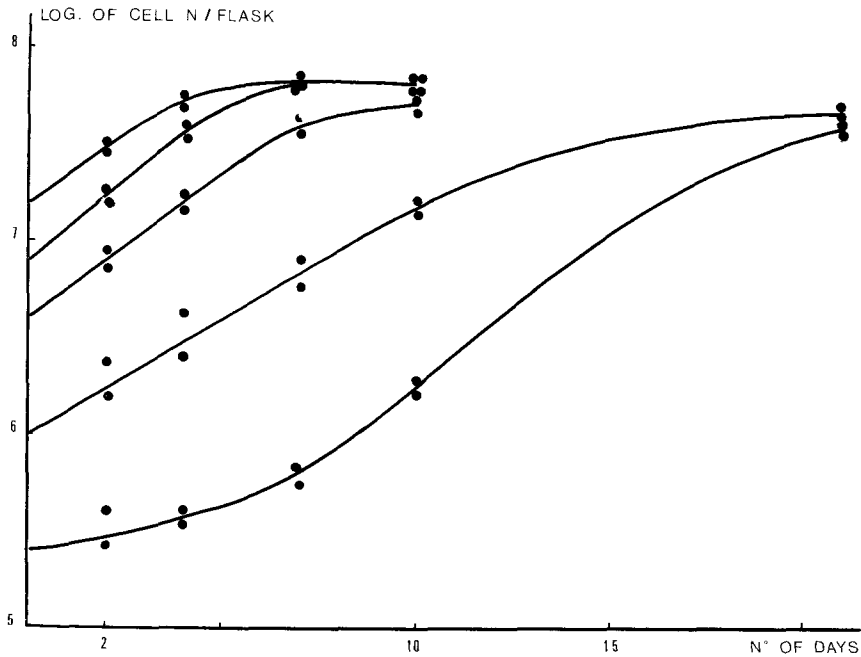


FIG. 3. — Cell growth as a function of the inoculum.

Culture flasks were seeded with 0.25, 1, 4, 8 and 16 million cells, respectively, and incubated at 30° C for 10 days for the 3 later inocula and 21 days for the 2 former. Cell productions in duplicate flasks were periodically harvested and enumerated as in figure 2.

Plating efficiency.

Before cloning, plating efficiency was 3 % (table II). In comparison, plating efficiency of the progeny cells of the selected microcolony reached about 30 %, which is considered critical for culture on a microcarrier [4].

TABLE II. — Comparison of the plating efficiency of EPC cells before and after cloning.

Nb of seeded cells per Petri dish	Nb of colonies obtained after 3 weeks		
	EPC, 62nd subcultures	Cloned EPC 8 subcultures post-cloning	Cloned EPC after thawing
10	1, 0	4, 3	ND
100	6, 2	31, 25	31, 18
1,000	23, 41	306, 282	207, 155

ND = not done.

Growth in semi-solid suspension culture.

No differences in cell growth were recorded when DEAE-dextran was added in the medium [13] but the density of cell colonies was noticeably higher in cloned EPC than in normal EPC (fig. 4).

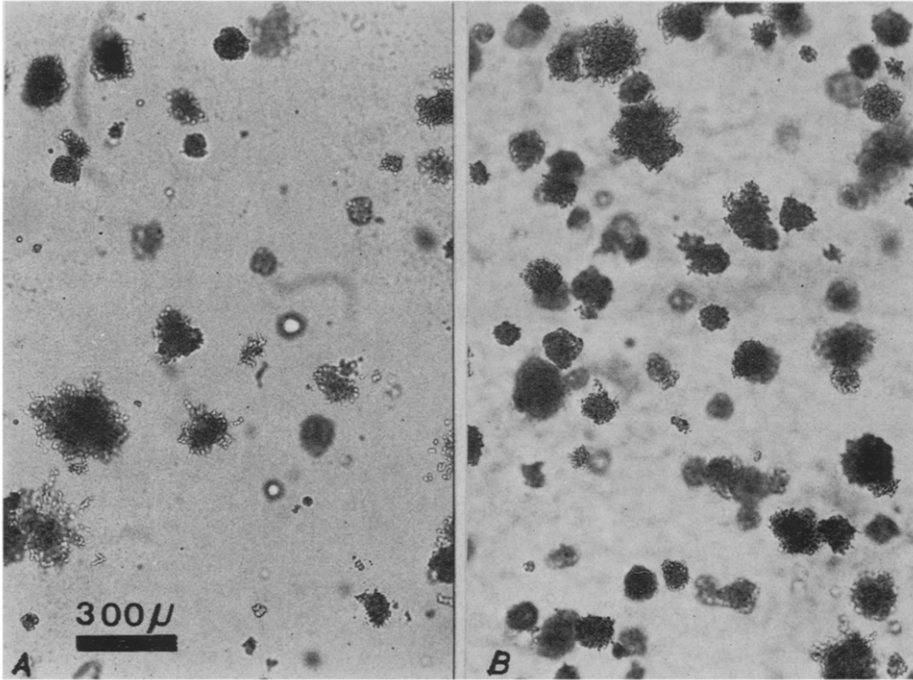


FIG. 4. — Fourteen-day old microcolonies of EPC cells in Stoker's medium containing 0.5 % agar. Seeding density was 10^4 cells/cm². A = uncloned cells; B = cloned cells (objective 4 x).

Virus propagation.

Among fish viruses tested, the Ab serotype of IPN virus was the only one which did not replicate onto EPC cells. The other viruses provided virus yields higher than 10^6 pfu/ml (table III). When used for plaque assays with salmonoid fish rhabdoviruses (*i. e.* Egtved and IHN viruses), the plaque number was usually higher in EPC cells than in salmonoid RTG-2 cells.

VSV Indiana was the only one of the warm-blooded vertebrate viruses able to induce a severe cytopathic effect within 24 h at 30° C following inoculation of the cells at infection multiplicities ranging from 1 to 10^{-3} pfu/cell.

On the contrary, no cytopathic effect was obtained with NDV or Reovirus, even after two successive blind passages; attempts at virus titration from the EPC-cell culture medium revealed no virus multiplication two days after the final inoculation.

TABLE III. — Susceptibility of EPC cells to viruses.

Virus	Inoculum Source	Multiplicity of infection (pfu/cell)	Incubation temperature (°C)	Onset of CPE (h)	Cell system for virus titration	Average virus titre (pfu/ml)
Egtved	FHM RTG	10^{-2} - 10^{-3}	15	40-44	EPC	$>10^8$
IHN	FHM RTG	10^{-2} - 10^{-3}	15	60	EPC	$>10^8$
SVC or PFR	FHM	10^{-2}	20	24	EPC	$>10^8$
Eel rhabdov. B12	Elver	10^{-2}	15	60	EPC	10^6
Eel rhabdov.	RTG	10^{-2}	20	40	EPC	$>10^8$
IPN Sp	RTG	10^{-2}	15	40	RTG	$>10^8$
IPN Ab	RTG	10^2 - 10^{-2}	15-20	none	RTG	$<10^1$
IPN VR 299	RTG	10^{-2}	15-20	30	RTG EPC	$>10^8$
VSV Indiana	BHK	10^{-1}	30	20	EPC	10^6
NDV	Chick embryo	1-10	30	none	Chick embryo	$<10^1$
Reovirus	L cells	1-10	30	none	L	$<10^1$

CPE = cell plating efficiency.

DISCUSSION

EPC cells originally isolated from carp skin hyperplastic lesions have now reached the 80th subculture, which represents at least 200 cell divisions of the primary cells. Thus, EPC cells may be considered as an established cell line. Like most established cell lines, they are transformed, and display an unusual number of chromosomes: 96 instead of 100 in the normal carp cell [14]. The degree of transformation attained by EPC cells allowed them to grow in soft agar medium, and was more marked after cloning. But their density-dependent growth inhibition still remained.

The failure to detect herpes virus in any of the examinations performed on EPC cell cultures does not necessarily imply its absence; however, reagents are unavailable for such detection, and the herpes virus observed in skin lesions always failed to grow *in vitro*. In the future, additional attempts will be made using indirect immunofluorescence with serum from a fish which has recovered from the so-called pox. In addition, EM screening of the cells revealed no mycoplasmic contamination, and attempts

at mycoplasma culture on PPLO agar (Difco) with cells previously subcultivated three times without antibiotics in the medium, remained negative. These cells constituted our frozen seeding stock (subculture 62).

Until now, all attempts at assessing the presence of a coronavirus in our EPC cells have been negative. On the basis of the absence of syncytia, this seems to indicate that our cell line was kept free of such contamination, which is also substantiated by the failure of immunofluorescence checking. The lack of any effect after inoculation with the OC49 virus strain can only be explained by the fact that this coronavirus type has not been involved in chronic infection of EPC cells reported elsewhere [9].

The susceptibility of EPC cells to fish viruses is similar to that of FHM cells; however, the latter also propagate echovirus 11 [6] and Sindbis virus [3], which have not been looked for in EPC cells.

Like all cell cultures of poikilothermic origin [25], EPC cells are able to grow and survive over wide temperature ranges and to retain high virus susceptibility as long as they are subcultivated when the growth curve reaches the beginning of the plateau. Thus, for cell maintenance, we recommend a seeding density of 3.2×10^5 cells/cm² which, in plastic vessels corresponds to a splitting ratio of 1/8-1/10. At such a ratio, cells undergo three divisions before the onset of saturation density and, when incubated at 15° C, this leads to one subculture per month, which constitutes an efficient means of maintaining both subculture frequency and division number at a low level.

During the period of our studies, two cyprinid fish cell lines were described. The first [15], which originated from goldfish *Carassius auratus* skin, appears to have a more narrow temperature range than EPC cells, and its splitting ratio is also significantly lower. Like EPC, the goldfish cell line exhibits a reduced chromosome number ($n = 94$). However, its virus susceptibility was not thoroughly checked, since it was shown to replicate only IPN and IHN viruses. The second, originating from *Carassius auratus langsdorffi* skin [8], also had an impaired chromosome number: $3n = 153$, instead of 156 (since this species is polyploid). At the time it was described, the cell line had undergone only 30 subcultures, and no other data were available. At present, no other cyprinid fish cell line is known [26].

If we compare EPC cell characteristics with those of cyprinids or other fish cell lines, it is evident that they offer good possibilities for virological investigation both in the field of fish disease and in comparative virology; their wide temperature growth range permits studies on thermo-sensitive events which might be impaired by the normal physiology of warm-blooded vertebrate cells.

Although this work deals primarily with EPC cells, it also represents the first published report on attempts to isolate the herpes virus, which had previously been seen [17] in carp pox tissue. This virus may have been responsible for the cell plating efficiency (CPE) in primary carp-pox cell cultures, but this CPE was not transmissible to primary carp cell cultures and FHM cells.

RÉSUMÉ

ISOLEMENT ET CARACTÉRISATION DE LA LIGNÉE CELLULAIRE
« EPITHELIOMA PAPULOSUM CYPRINI » (ECP) DE LA CARPE

La séparation enzymatique des cellules d'une lésion cutanée hyperplasique de la carpe induite par un virus de type herpès, a conduit à l'obtention d'une lignée cellulaire épithélioïde appelée EPC (*Epithelioma papulosum cyprini*) qui a maintenant atteint sa 80^e subculture. Cette lignée se développe entre 15 et 33° C, survit sans inconvénients pour son comportement ultérieur entre 7 et 10° C et dégénère rapidement à 37° C. A 30° C, l'ensemencement de 3×10^5 cellules/cm² dans du milieu de Stoker, additionné de 10 % de phosphate de tryptose et de 10 % de sérum d'embryon de bovin, est suivi d'un décuplement du nombre initial des cellules en 6 à 7 jours, qui conduit à la densité de saturation. Le nombre moyen de chromosomes est de 96 au lieu de 100 dans les cellules primaires de carpe, et leur transformation leur permet de se développer en suspension dans un milieu gélifié. Le virus herpétique présent dans la lésion d'origine n'a jamais pu être retrouvé au cours des contrôles effectués, et l'infection locale qu'il induit chez son hôte est d'ailleurs bénigne.

Les cellules EPC sont sensibles à tous les rhabdovirus de poissons d'eau douce actuellement décrits ainsi qu'à deux des sérotypes du virus de la nécrose pancréatique infectieuse (Sp et VR299) et au virus de la stomatite vésiculeuse de type Indiana.

Leur large plage thermique de croissance et leur sensibilité à de nombreux virus en font un matériel de choix en pathologie virale des poissons comme en virologie comparée.

MOTS-CLÉS : *Epithelioma papulosum cyprini*, Variole de la carpe, Virus herpès, Culture cellulaire; Caryotype, Rhabdovirus, Pathologie des poissons.

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