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PECULIARITIES OF THE VIRUS-HOST CELL RELATIONSHIP IN A CALF KIDNEY CELL LINE PERSISTENTLY INFECTED WITH MEASLES VIRUS

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Summary. — Persistent infection with measles virus established in a calf kidney cell line differed in some features from other measles virus carrier systems. After a variable life span, ranging from 50 to 1100 days, six of the chronically infected cell lines showed an evolution towards lytic infection. Only one of these lines, the K-2 cell line, exhibited marked morphological, growth and chromosomal alterations suggesting in vitro cellular transformation. The transformed K-2 cells released infectious measles virus with several modified biological parameters. Persistent infection was not due to the accumulation of thermosensitive mutants or defective particles. The role of the host-cell factor and of the selection of the virus population during its passage in RV cells are discussed.

Key words: measles virus; *cell culture*; *persistent infection*

Introduction

Rustigian's (1962) experiments, demonstrating for the first time persistent measles virus infection in vitro, seemed to have no clinical significance at that time. The involvement of measles virus in the aetiopathogenesis of subacute sclerosing panencephalitis (SSPE) stimulated the interest in the study of experimental chronic infection both in vitro and in vivo.

Persistence of measles virus in various cell types cultivated in vitro (Rustigian, 1966; Norrby, 1967; Minagawa, 1971; Knight *et al.*, 1972) is marked by certain common features: the cytopathic effect (CPE) and yield of infectious virus are reduced or absent, the yield of haemagglutinin also decreases, haemadsorbing cells become less numerous, while the percentage of antigen-bearing cells, revealed by immunofluorescence, is high (85–95 %).

Different mechanisms varying from one system to another have been suggested for the establishment and the maintenance of persistent intracellular infection (Fraser and Martin, 1978). The study of the virus-host cell relationship within the chronic carrier state has supplied very little informa-

tion concerning the effects of persistent measles virus infection on cellular morphology and cytogenetics. The cellular substrate in which these infections were initiated — usually heteroploid cells — represented a serious handicap from this viewpoint.

Relying on the limited susceptibility to measles virus of bovine kidney cells, we studied the possibility of establishing calf kidney cell lines chronically infected with measles virus (Sorodoc *et al.*, 1977) and some characteristics of these lines (Sorodoc *et al.*, 1978, 1979).

We describe here some peculiarities of the virus-host cell relationship in our carrier system.

Materials and Methods

Cell cultures. The following cell cultures were used: (*1*) R_4V calf kidney cell line and (*2*) the $R_{17}CA$ *Cercopithecus aethiops* kidney cell line, both obtained in the "St. S. Nicolau" Institute of Virology, Bucharest; and (*3*) calf kidney cell lines (RVCo-1, RVCo-4, RVCo-5, RVCo-6, K-2) persistently infected with measles virus, obtained as described previously (Sorodoc *et al.*, 1977). The cell lines were maintained by EDTA-trypsin treatment at 7—10 days intervals and at a split ratio of 1 : 2 or 1 : 3 in IC-65 growth medium (Dr. I. Cantacuzino Institute, Bucharest) supplemented with 10 % calf serum and 2.5 % N-16 nutrient solution. The maintenance medium contained Earle's and Hanks' solutions, lactalbumin hydrolysate, 2 % calf serum, and 2.5 % N-16 nutrient solution.

Viruses. The following measles virus (genus *Morbillivirus*) strains were used: (*1*) the wild autochthonous Co-69 strain; (*2*) the Co-69/p. i. strain, recovered from the persistently infected K-2 cell line after 40—46 subcultures at 37 °C; and (*3*) the attenuated Schwarz strain from a Schwarz vaccine (Rouvax batch M 050307/1976), passed twice in chick embryo fibroblasts.

Infectivity assays. Culture fluids or materials obtained after 3 cycles of freezing and thawing of cells detached with EDTA-trypsin, clarified by centrifugation, were adsorbed for 2 hr on to susceptible $R_{17}CA$ cells and incubated at 37 or 40 °C. $TCID_{50}$ was estimated by Kärber s formula after 18 days of incubation with repeated medium changes.

Morphological study. The morphological aspects of the cells were followed by direct microscopic examinations of the culture tubes or on methanol-fixed and haematoxylin and eosin stained preparations.

Cell growth rate. Tubes were seeded with 1 ml cell suspension of known concentration in growth medium containing different amounts of calf serum (10 %, 0.1 %). The cells were counted daily for 5 days: each determinaton consisted of 3 cell counts done in a pool from 3 tubes. Mean values and standard deviations were calculated.

Haemagglutination and haemadsorption tests were carried out as described by Norrby (1967) and Sorodoc (1971), using *C. aethiops* red blood cells (RBC). The percentage of haemadsorption-positive cells was determined by examination of 500—600 cells.

Plaque tests were performed in $R_{17}CA$ cells incubated at 37 °C with 1.25 % Noble (Difco) agar. The size of plaques ("S") marker) was examined after 5—6 days on staining with 0.01 % neutral red or 1 : 10 Giemsa solution.

Replication capacity at 40 °C (rct $_{40}$). The rct_{40} marker was evaluated as recommended by Hozinski *et al.* (1968).

Thermosensitivity was determined by maintaining a virus suspension diluted 1 : 5 in Earle's solution at 50 °C for 15, 30 and 60 min. The T_{50} marker (Hozinski *et al.*, 1968) and thermal inactivation rate were estimated.

Assay for an interferon-like inhibitor. Medium from persistently infected cell lines and from the control culture were collected on days 2, 5, and 7 after seeding. Samples were processed as described by Merigan *et al.* (1966). Twofold dilutions of the samples were applied to normal R_4V cells for 24 hr at 37 °C; subsequently vaccinia virus (100 and 500 $TCID_{50}$) was inoculated and the protection against vaccinia infection was determined.

Chromosome analysis was performed on the K-2 cells and R_4V control cells. Colcemid solution was added to the medium at a final concentration of 0.5 μg/ml. After 2—3 hr at 37 °C, the cells

Table 1. Establishment and evolution of calf kidney cell lines persistently infected with measles virus

Virus strain	Cell line (passage level)	Persistently infected cell line obtained	No. of passages in vitro	Life span (days)	Stage of in vitro evolution; morphological and growth characteristics
wild Co-69	R_4V (14)	RVCo-1	1–32	21–285	Cell monolayer; CPE − → +
			33	769	Cell monolayer; CPE +
			34–35	770–790	Total cell lysis by CPE +++
	R_4V (22)	RVCo-2	1–21	11–215	Cell monolayer; CPE − → +++
			22	216–248	Cell lysis by CPE +++ ("crisis")
		K-2 (selected line)	25	269	Overcoming of the "crisis"; cell islets
			26–175	270–1500*	Stabilized cell monolayer with morphological and growth alterations
	R_4V (31)	RVCo-4	1–40	14–345	Cell monolayer; CPE − → +
			41–48	346–436	Cell monolayer; CPE +++ → total cell lysis
			27–34	172–1140*	Cell monolayer; CPE ++; islets of surviving cells
	R_3V (7)	RVCo-5	1–26	7–172	Cell monolayer; CPE −
			27–28	173–289	Cell monolayer; CPE + → +++ and cell lysis; cell recovery
			29–31	210–354	Unstabilized cell monolayer; progressive CPE +++ → total cell lysis
	R_4V (11)	RVCo-6	1–42	7–282	Cell monolayer; CPE −
			43–47	283–347	Cell monolayer; CPE + → +++; total cell lysis
attenuated L-16	R_4V (14)	RVL-1	1–8	10–78	Cell monolayer; CPE − → +
			9–10	79–100	CPE +++ → total cell lysis
	R_4V (22)	RVL-2	1–6	10–45	Cell monolayer; CPE − → +
			7	50	CPE +++ → total cell lysis

* To date.

were detached with EDTA-trypsin and subjected to hypotonic treatment (3 parts distilled water were added to 1 part cell suspension) at 37 °C for 20 min. The cells were then centrifuged at 1000 rev/min and subjected to three successive cold fixations with methanol-glacial acetic acid. Smears preparations were air-dried and stained with aqueous Giemsa solution.

Table 2. Biological characteristics of calf kidney cell lines persistently infected with measles virus

Persistently infected cell line	Passage level	Life span (days)	Virus yield ($TCID_{50}$/ml)		CPE	Haemadsorption (%)
			cell-associated	cell-free		
RVCo-1	26	226	2.5	1.5	−	0
	29	263	4.75	4.75	+	20
	34	770	4.5	3.75	+	20−30
RVCo-2	4	34	5	3.5	+	10−20
K-2	22	162	4.75	2.5	+++	90
	31	295	3.75	2.75	+	80
	34	350	6	4.25	+	90
	56	530	5.5	4	+	70
	89	765	6.25	4.5	+	90
	125	1083	5.75	4.5	+	80
	146	1230	5.5	3.5	+	80
RVCo-4	23	144	3	0	−	0
	43	396	4.5	2	++	30
RVCo-5	5	47	2.5	1.75	−	0
	23	165	5	3.5	±	0
RVCo-6	22	156	4	2	−	0
	34	229	4.5	2	−	0
RVL-1	8	60	4.5	2.5	+	10
RVL-2	6	52	4.75	5.25	+	10

Electron microscopy. The infected K-2 and control R_4V cells from 3-day-old cultures were fixed in situ with a 2 % glutaraldehyde solution in phosphate buffered saline (PBS) for 90 min, scraped off and centrifuged at 800 × g for 10 min. Cell pellets were post-fixed with 1 % osmium tetroxide for 2 hr, mixed with 2 % agar, dehydrated with ethanol and acetone and embedded in Epon R. Sections were cut with an LKB or Porter Blum ultramicrotome, stained with uranyl acetate followed by lead citrate and examined in a Philips EM 201 and SEM-3 electron microscope.

Results

Evolution of calf kidney cell lines persistently infected with measles virus

The in vitro evolution of the persistently infected cell lines was different (Table 1). Chronically infected cell lines initiated with the attenuated L_{16} strain showed a rapid evolution to lytic infection, after a life span of 50−100 days. Likewise, the cell lines initiated with the wild Co-69 measles virus strain (RVCo-1, RVCo-5, RVCo-6) also showed an evolution towards total cellular

lysis, after a life span ranging from 350—800 days by development of a CPE characteristic of measles virus. In spite of a marked CPE exhibited after passage 29, the persistently infected cells of the RVCo-4 line could be maintained in the form of small surviving cell islets for more than 1100 days.

The evolution of the RVCo-2 cell line in vitro was peculiar. During the 21 passages, measles virus-specific lesions appeared in delimited areas of the epithelioid monolayer and spread quickly afterwards, so that at passages 22—24 most of the cells were destroyed. By the "cell survivor" method we established a selected cell line, termed K-2, which could be subcultured for a long time. The split ratio was from 1 : 3 to 1 : 6. This persistently infected cell line has preserved the same morphological and growth characteristics, beginning with passage 26 (at 270 days of life) and throughout the 175 passages performed so far (i. e. over a life span of 1500 days).

Biological, morphological and growth characteristics of the calf kidney cell lines persistently infected with measles virus

In our persistently infected calf kidney cell lines, measles virus was recovered in high titres from both infected cells and the culture fluids (Table 2). The amount of infectious virus released into the culture fluid always was inferior to that detected in infected cells. The highest yield of infectious virus was recorded in K-2 cells (10^4—$10^{6.25}$ $TCID_{50}$/ml). In spite of the significant yield of infectious virus the proportion of cells binding *C. aethiops* RBC was low (10—30 %) in most of the infected cell lines. Only in the RVCo-2 cell line there was a great increase in the incidence of haemadsorption-positive cells. The proportion of RBC-binding K-2 cells ranged from 70 % to 90 %.

Neither free nor associated haemagglutinin could be detected at any passage level.

At the first passages, the morphology and growth rate of infected cell lines were indistinguishable from those of uninfected control cells.

After a various number of passages, depending on the type of infected line, there appeared a limited number of small syncytia with 4-5 nuclei and cytoplasmic and nuclear inclusions. With time, on continuous passaging, there was an extension of the viral CPE and total cellular lysis occurred.

The K-2 cell line showed marked morphological alterations: a predominantly epithelial polymorphous aspect with small syncytia, giant cells and cytoplasmic vacuoles. On day 7 after seeding, foci of multilayered cell proliferation (Fig. 1) with marked nuclear pleomorphism as regards size and shape appeared within the monolayer. Large and aberrant nuclei, sometimes surrounded by condensed and hyperchromic cytoplasm, could be observed (Fig. 2). Nuclear inclusions were absent.

The control represented by uninfected R_4V cells constantly showed a homogeneous regular aspect of the cell monolayer (Fig. 3). After 80 passages there was a tendency of transition towards epithelioid cells, but the homogeneity of the monolayer was preserved.

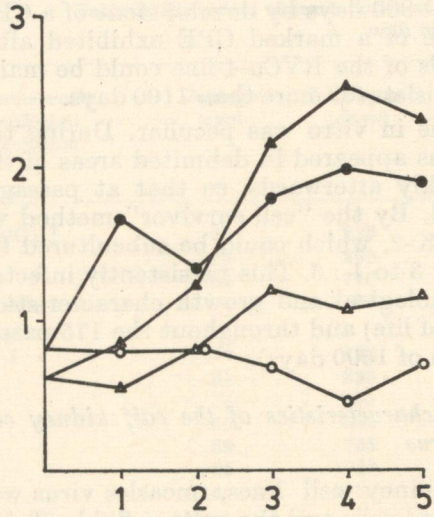

Fig. 7.
Cell growth rate
● R_4V (10 % calf serum)
○ R_4V (0.1 % calf serum)
▲ K-2 (10 % calf serum)
△ K-2 (0.1 % calf serum)
Abscissa: days after seeding; ordinate: cel count per ml × 10^{-5}

Electron microscopy of K-2 cells at the 155th passage revealed the presence in the cytoplasm of aggregates of granular filaments, in the perinuclear zone and under the cell membrane (Figs 4 and 5). The diameter of the cytoplasmic granular filaments measured from 20 to 30 nm. This structure was never seen in the nucleus. Numerous pleomorphous particles with diameters from 100 to 600 nm were found budding at the cell surface and in the intercellular space. Most of these particles contained different amounts of nucleocapsid tubules and had an envelope with discontinuous "fuzzy" material (Fig. 6).

At higher passage levels, the cell growth rate of most of the persistently infected cell lines was slower as compared with control cultures. By contrast, the K-2 cell line was characterized by rapid growth. The cell growth rate increased progressively, irrespective of the calf serum concentration used (Fig. 7). At calf serum concentration of 0.1 %, the number of R_4V cells decreased, whereas that of K-2 cells doubled. At 96 hr after seeding, at a 10 % calf serum concentration the number of cells increased more than 4-fold in the K-2 cell line and only 2.5-fould in R_4V cells.

All attempts at detection of an interferon-like inhibitor in the culture fluids from unifected and infected cell lines were negative.

Chromosome analysis of the K-2 cells

At passages 55 and 136, K-2 cells showed severe changes in the distribution of chromosome numbers, as well as morphological and structural alterations as compared with the uninfected R_4V line analysed at passages 48, 70 and 104. The R_4V cell line was predominantly hypodiploid. Some changes as against the normal bovine metaphase were recorded, consisting in the

Table 3. Biological characteristics of the Co-69, Co-69/p.i. and Schwarz strain of measles virus

Virus strain	Infectivity (log $TCID_{50}$/ml) 37 °C	40 °C	rct_{40}	T_{50}	HAU/ml	Plaque size "S" (%) pin-point	≦1 mm	>1 mm	CPE
Co-69	6.6	6.3	+	+	64	15	25	60	rapid, syncytia ++++, inclusions ++++
Co-69/p.i.	4.1	2.1	−	±	0−2.6	90	10	0	slow, spindles +++, syncytia +, inclusions +
Schwarz	4.5	2.5	−	−	0	90	10	0	slow, spindles +++, syncytia +, inclusions +
Co-69/p.i.*	5	3.75	±	+	0	72	25	3	slow, spindles +++, syncytia +, inclusions +
Schwarz*	3.75	2	−	−	0	94	6	0	slow, spindles +++, syncytia +, inclusions +

* After 10 passages in the simian substrate ($R_{17}CA$).

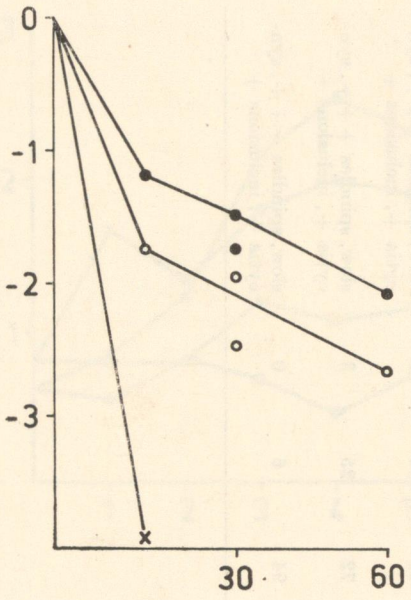

Fig. 11.

Inactivation rate at 50 °C of Co-69 (●), Co-69/p. i. (○) and Schwarz (×) measles virus strain. Abscissa: $-\log N_0/N_t$ (N_0 = initial infectious titre; N_t = infectious titre after inactivation); ordinate: time of inactivation (min)

appearance of 1—5 atelocentric marker chromosomes (Fig. 8). The proportion of satellite-type constrictions was reduced (10 %).

In the K-2 cell line, 62 % of the metaphases were hypotetraploid and 28 % tetra- or hypertetraploid. All chromosomes represented new chromosome types (Figs 9 and 10). Structural alterations were frequent: secondary constrictions (48 %), satellite-type constrictions (29 %) and achromatic gaps (22 %).

Biological characterization of the virus recovered from the K-2 cell line

In an attempt to identify a modification or selection of the virus population during its passages in RV cells, some biological markers of the wild Co-69, Co-69/p. i. and attenuated Schwarz strains were compared.

The 40/37 °C ratio was —0.3 log for the parental Co-69 strain and —2 log for the Co-69/p. i. and Schwarz strains. The Co-69 strain was $rct_{40}+$, while both the Co-69/p. i. and the Schwarz strain were $rct_{40}-$ (Table 3).

The Co-69/p. i. and Schwarz strains did not agglutinate *C. aethiops* RBC, while the parental Co-69 strain was highly haemagglutinating.

The plaques formed by the Co-69/p. i. and Schwarz strains were small, predominantly pin-point, while most of the plaques formed by the parental Co-69 strain had a diameter > 1 mm.

The CPE produced by the Co-69/p. i. and the Schwarz strains had a latency of 5-6 days and a slow progression, lasting for 14-18 days. It was characterized by spindle-shaped forms and rare small syncytia. Rare nuclear and cytoplas-

mic inclusions were noted on days 7-8. The parental Co-69 strain caused a rapid CPE within 24-48 hr with large syncytia containing 20-100 nuclei; numerous inclusions were present in almost all cells after 3-4 days. The CPE was total on days 8-10.

Inactivation rates at 50 °C for the three strains studied (Fig. 11) were different. During the first 15 min, complete inactivation of the Schwarz strain occurred; the infectious titre of Co-69/p. i. virus decreased by 1.85 log units after 15 min and by 2.85 log units after 60 min. The titre decrease of the parental Co-69 strain was 1.1 and 2.3 log units after 15 and 60 min, respectively. According to Hozinsky's formula, Co-69/p. i. is $T_{50}\pm$, while Co-69 $T_{50}+$ and Schwarz $T_{50}-$.

After two successive clonings from small plaques (< 1 mm), the Co-69/p. i. and Schwarz strains were carried through 10 passages in $R_{17}CA$ cells. The Co-69/p. i. strain became $rct_{40}\pm$, $T_{50}+$, while the Schwarz strain remained $rct_{40}-$ and $T_{50}-$. However, the aspect, intensity and evolution of the CPE remained unchanged for both strains. The "S" marker remained unaltered for the Schwarz strain and showed a mino ralteration for Co-69/p. i. virus consisting in the appearance of plaques > 1 mm (3 %).

Discussion

Several mechanisms have been proposed to explain the development of the persistent state in virus infections. In the present experiments a host-cell factor played an important role in the establishment of the persistent infection with measles virus. The cell substrate used by us is little susceptible to measles virus infection (Schwarz and Zirbel, 1959; Matumoto *et al.*, 1961; Aderca *et al.*, 1972). Cellular resistance to measles virus and appearance of permissive cells within the mass of resistant cells seems to govern the initiation of the chronic infection. The percentage of infected cells and virus yield during the initial infection of RV cells were low, but after some passages an increased yield of infectious virus, especially of extracellular virus, and an extension of the CPE were observed.

The mechanism of cellular resistance is not clear. Interferon or interferon-like substances could not be detected in the culture fluid, but the involvement of an intracellular interferon-like factor cannot be excluded.

The ability of the measles virus to establish and maintain persistent infection in RV cells is not solely due to a host-cell factor. The evolution of the persistent infection varied depending on the virus strain inoculated. The evolution towards lytic infection was more rapid in cell lines persistently infected with the attenuated L-16 strain. This suggests that the role of the measles virus strain in the measles virus — calf kidney cell relationship cannot be neglected.

The study of the selected K-2 cell line, persistently infected with measles virus and subcultured for a long time in vitro, allowed us to make some considerations concerning the possible in vitro transforming action of measles virus and the maintenance of the carrier state in the presence of infectious particles.

As compared with the original RV line, the K-2 cell line showed marked alterations in cytogenetic, morphological and growth characteristics. The factors possibly involved in determining this transformation are: (*a*) the measles virus, whose transforming capacity has not been confirmed so far. Webb *et al.* (1971) reported cell transformation in human foetal brain cultures during a prolonged primary infection with the attenuated Schwarz strain. Cell transformation in nerve cell cultures from SSPE patients was observed by Katz *et al.* (1969), who correlated it with the presence in the brain tissue of a transforming agent other than measles virus; (*b*) the bovine cell substrate known to exhibit cytogenetic and morphological alterations in the course of passaging, sometimes from very early stages (Lithner and Pouten, 1966; Nelson-Rees *et al.*, 1967).

The morphocytogenetic particularities and the growth rate of the R_4V cell line at passages close to that of the K-2 cell line ruled out the possibility of a spontaneous transformation. The morphology and growth rate of the R_4V cells remained unchanged during serial passages and their cytogenetic aspect presented some changes (the appearance of marker chromosomes), but not so marked as those in the K-2 cell line.

Cell transformation occurred only in one of the seven persistently infected cell lines studied. It is clear that if measles virus were able to induce transformation in vitro, its transforming potential is rather limited. The possible existence of another latent transforming agent in the bovine cells and its activation by measles virus, which would have played the role of a helper, cannot be excluded.

In the K-2 cell line, the transforming process was not accompanied by a modification in the synthesis of infectious virus, which was released into the culture fluid in considerable amounts.

The nature of the virus in this carrier culture was also investigated and it proved to be a variant of the parental Co-69 strain, with characteristics of an attenuated strain. The Co-69/p. i. virus was characterized by a late strand-forming CPE, a lower 40°/37° ratio, undetectable HA activity, predominantly pin-point plaques and a medium thermosensitivity. These biological properties resembled those of the U-P variant of measles virus described by Norrby *et al.* (1970) or of the purified UP-SP 4 clone described by Chiarini *et al.* (1976). These authors observed that these variants obtained by successive undiluted passages induced persistent infection.

Some of the biological characteristics acquired by the Co-69 strain remained stable during ten passages in a simian substrate (cytopathogenicity, HA activity, size of plaques). The lack of restrictive replication at 40 °C shows that the Co-69/p. i. variant is not thermosensitive.

Selection of certain characteristics during serial subcultivation in the bovine substrate under the action of cellular factors represents a possible mechanism of the appearance of this laboratory variant of virus.

In our system, the nucleocapsids were transmitted to progeny cells by cell division but, on the other hand, many of them had the chance of being incorporated into budding particles and became infectious virus particles.

In K-2 cells, the cytoplasmic granular filaments were associated with active budding, virus particles with incomplete envelopes and containing variable amounts of nucleocapsids were present and the yield of infectious virus was high.

The selection — at a low rate — of a non-susceptible cell population, may have played a role in the maintenance of the carrier state in RV cells, but a modified cytopathogenicity of virus particles may have also been partially responsible. The limited proportion of electron-translucent particles, seen in K-2 cells, confirmed that defective-interfering particles were not involved in the mechanism of this persistent infection.

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Explanations of Micrographs (Plates XIX—XXIII):

Figs 1 and 2. K-2 cells, passage 55 (7 days); *1* — zone of multilayered cell proliferation, × 144; *2* — large and aberrant nuclei, × 270. Haematoxylin and eosin.

Fig. 3. R_4V cells, passage 45 (7 days). Homogeneous regular aspect of the cellular monolayer; spindle-shaped cells with oblong, oval nuclei. Haematoxylin and eosin, × 270.

Fig. 4. Part of a K-2 cell. Large aggregate of granular filaments in the cytoplasm, perinuclear zone. × 19,000.

Fig. 5. Part of a K-2 cell. Detail of intracytoplasmic granular filaments. × 45,000.

Fig. 6. Surface area of a K-2 cell. Pleomorphous particles in the budding process and in the intercellular space. The particles have an envelope with discontinuous "fuzzy" material and contain variable amounts of nucleocapsids. × 45,000.

Fig. 8. Metaphase in an uninfected calf kidney cell (R_4V); passage 104. Marker chromosomes; hypodiploidy.

Figs 9 and 10. Metaphase in K-2 cells at passage 136. Heteroploidy; all chromosomes are new chromosome types (markers). *9* — satellite type constriction, *10* — dicentric chromosome.