ENDOSYMBIOTIC RELATIONSHIP BETWEEN ANIMAL VIRUSES AND HOST CELLS

A Study of Rabies Virus in Tissue Culture*

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Plates 98 to 100

(Received for publication, August 3, 1964)

Persistent infection of various tissue culture systems with rabies virus has been described in several communications (1–3). In the course of these investigations, it became apparent that, depending on the tissue culture system used and on the stage of cultivation, rabies antigen may be present in all cells of the culture but growth of the infected culture does not seem to be impaired. This situation seems to be particularly characteristic for a rabies-infected rabbit endothelium cell line (1). This paper presents an analysis of the virus–host cell relationship in such a system.

Materials and Methods

Virus.—The challenge virus standard (CVS),[1] a fixed rabies virus propagated in mouse brain (MB-CVS), was originally used to infect the rabbit endothelium (RE) cell system (1) and the Pitman-Moore (PM) strain of fixed rabies virus propagated in rabbit brain (RB-PM)[1] was used in the interference experiments. The two virus pools were maintained at −70°C as a 20 per cent suspension of the infected brain tissue in 50 per cent normal calf serum in distilled H_2O. A strain of vesicular stomatitis virus (VSV) propagated in MK-2 cells in tissue culture was also used in the interference experiments.

Tissue Cultures.—Monolayer tissue cultures of rabbit endothelium cells (4) and rabies virus–infected rabbit endothelium cells (RE-CVS) (1) were propagated in milk dilution bottles and subcultivated twice weekly. The nutrient medium consisted of modified Eagle's basal medium (5) in Earle's balanced salt solution supplemented with 10 per cent inactivated calf serum and containing 25 ml of 5.6 per cent sodium bicarbonate, 10^5 units of penicillin, and 10^5 μg of streptomycin or 5×10^4 mg of aureomycin per liter.

For cytological observations and immunofluorescent antibody staining (FA) described below, the cells were grown in the same medium on coverslips in Petri dishes and incubated at 37°C in a humidified CO_2 incubator.

A stable line of neonatal hamster kidney fibroblasts, BHK-21, clone 13, referred to as

* This work was supported, in part, by United States Public Health Service Research Grant AI-02954 from the National Institute of Allergy and Infectious Diseases. and a grant from the World Health Organization.

[1] Supplied by the National Institutes of Health, Bethesda.

C-13 (6), propagated in the same nutrient medium was used for some of the *in vitro* titrations and for the infectious center experiments (see below).

Titration of virus by FA in tissue culture: 10-fold dilutions of the virus were prepared for titration in nutrient medium and 1 ml of each dilution was mixed with 5 ml of a C-13 cell suspension containing 5×10^5 cells, which were plated out in Petri dishes containing coverslips and incubated at 37°C for 6 days and stained. The highest dilution of virus causing staining of cells by FA was considered the titration end-point.

Infectious centers: C-13 cells were grown in Petri dishes (50 mm) in monolayers over coverslips and seeded with a suspension of RE-CVS cells containing approximately 1000 cells per dish. Taking into consideration the dimensions (11 x 22 mm) of the coverslips, approximately 100 RE-CVS cells were seeded for each coverslip. After 18 hours of incubation, one coverslip was removed from each Petri dish and stained by FA, and the number of dispersed fluorescing RE-CVS cells were counted. After 6 days' incubation the other coverslips were removed and stained by FA, and the number of fluorescing cell aggregates containing more than 10 cells counted.

Plating efficiency: Five hundred RE and RE-CVS cells were plated out into 50 mm plastic Petri dishes for each series of tests for plating efficiency. The plates were cultivated for 16 days, the viable colonies in each plate were counted, and the number averaged to determine the final result for each particular series.

Cytological preparations: Actively proliferating cultures were selected for chromosome analysis and treated according to the method described by Saksela and Moorhead (7).

Autoradiography: Cultures of RE and RE-CVS cells, grown on coverslips in Petri dishes, were exposed to 2 μc/ml of H_3-thymidine (specific activity 3 c/mmole). At different times after exposure, ranging from 30 minutes to 7 hours, the coverslips were washed several times in phosphate-buffered saline (PBS) and fixed in Carnoy's solution followed by Feulgen's reaction. They were coated with NTB 3 emulsion (Eastman Kodak Company, Rochester, New York), exposed in the dark at 4°C for 10 days, the film developed in D-19 solution and fixed with acid. To ensure good cellular detail, the cultures were then stained with 5 per cent toluidine blue at pH 3.5.

In another series of experiments, the cultures of control and infected cells were exposed to 6 μc/ml of H_3-uridine (specific activity 4 c/mmole) for periods varying from 30 minutes to 28 hours. The coverslips were washed in PBS, fixed in Carnoy's solution for 30 minutes, and treated with 2 per cent percholoric acid for 40 minutes at 4°C to remove soluble nucleotides. Half of the coverslips in each group were treated with RNase (Worthington Biochemicals Corp., Freehold, New Jersey) 60 μg/ml in veronal acetate buffer, pH 7.8, for 2 hours at 37°C. Finally, the coverslips were covered with NTB 3 emulsion, exposed in the dark for 20 days, and treated as in the previous experiment.

Antirabies Sera.—Three types of sera were used:

(*a*) Serum obtained from a man who had, in the past, undergone an antirabies Pasteur treatment (Fermi type of vaccine) and had, in addition, received 3 booster inoculations of Flury HEP virus. This serum which had a neutralization titer of 1/380 in mice is referred to here as Human W.

(*b*) Serum from a man who had received 3 injections of rabies duck embryo vaccine followed 1 year later by 1 booster inoculation of the same vaccine. This serum had a neutralization titer of 1/830 and is referred to here as Human F.

(*c*) Antirabies gamma globulin prepared in horses at the Metchnikoff Research Institute, Moscow, U.S.S.R.[2] with a neutralization titer of 1/1000 in mice (referred to here as Horse P).

Complement.—Blood obtained from young adult hamsters by heart puncture was stored

[2] Obtained through the kindness of Dr. Martin Kaplan of the World Health Organization.

overnight at 4°C. The serum was then centrifuged off, immediately frozen, and used in a 1:10 dilution.

Microscopic Observations.—

Phase contrast: Coverslip preparations were mounted in closed chambers and observed under phase contrast optics.

Immunofluorescent antibody staining (FA): Cells on coverslips were stained directly with antirabies globulin conjugate,[3] using the technique of Coons and Kaplan (8), as modified by Goldwasser *et al.* (9).

May-Grünwald–Giemsa staining: Coverslips were washed twice in PBS, fixed in Bouin's fixative for 10 minutes, stained with May-Grünwald for 15 minutes and then for 30 minutes with a 1/30 Giemsa solution in buffered distilled water (pH 6.8). They were then processed following routine procedure for preparation of permanent mounts.

Inoculation of Experimental Animals.—

Mice: For titration purposes, 3- to 5-week-old Swiss white mice were injected intracerebrally with serial dilutions of virus. The end-points were calculated according to the method of Reed and Muench (10). For immunization purposes, 5-week-old mice were injected intraperitoneally with 0.25 ml of an undiluted suspension of RE-CVS. Three weeks later these mice were challenged either intramuscularly with 0.1 ml of a 1:30 dilution or intracerebrally with 0.03 ml of serial dilutions of MB-CVS pools. Non-vaccinated mice of the same age were used as controls.

Rabbits: Five-day-old and young adult rabbits were inoculated intracerebrally with 0.05 ml of a cell free extract from RE-CVS cells.

RESULTS

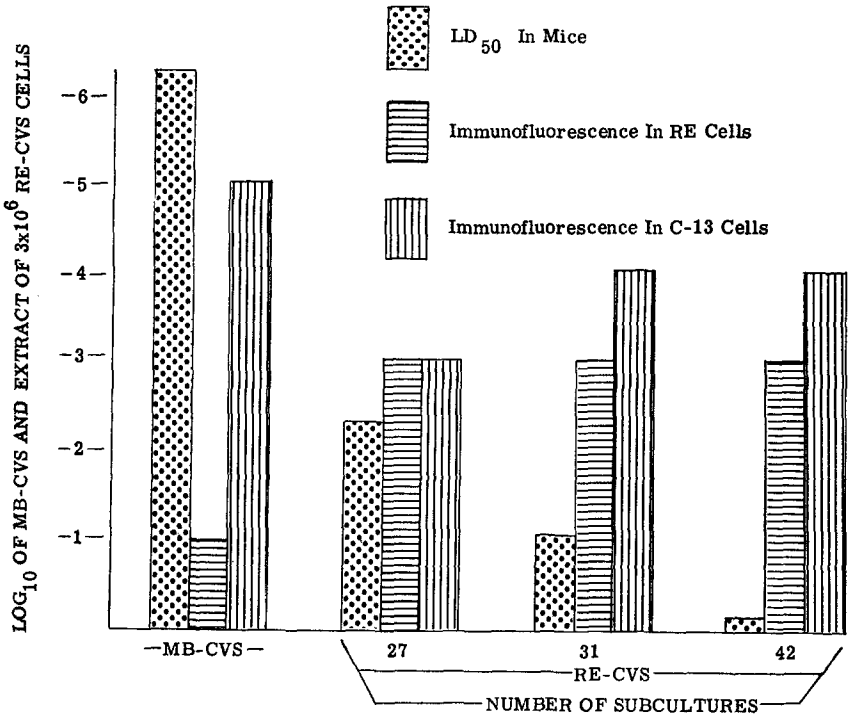
Immunofluorescence of Cells Containing Rabies Antigen.—RE cells exposed in suspension to MB-CVS at an input multiplicity of 5 can be grown as monolayers in tissue cultures and maintained for several consecutive transfers without evident degenerative changes (1). Through ninety-three transfers of the infected cells, there has been no evidence of cytopathic effect (CPE). The presence of viral antigen in the cultured cells was determined by staining RE cells grown on the coverslips with fluorescein-labeled antiserum.

Three days after exposure to the virus pool (MB-CVS), small bright fluorescent granules were observed dispersed throughout the cytoplasm and in a few RE cells the granules were concentrated in the perinuclear zone. On the 7th day, the granules increased in size and formed small, round, or oval-shaped, inclusion-like masses, but the percentage of cells showing fluorescence remained low (1 per cent) through the 10th day of cultivation. During subsequent transfers, the number of RE cells showing the presence of intracytoplasmic antigen increased rapidly from the level of 25 per cent at the third subculture to 100 per cent at the fourth subculture. The persistence of viral antigen in all the cells of the RE-CVS cultures was observed throughout the

[3] Antirabies gamma globulin conjugate supplied by the National Rabies Laboratory, Atlanta, Georgia.

15 months' cultivation period, corresponding to 93 cell transfers (Fig. 1). The cytoplasmic inclusions were the same size and shape as those in the cells primarily infected with the MB-CVS pool and as shown in Figs. 2 to 5, they were observed in a number of cells in various stages of mitosis.

After May-Grünwald–Giemsa staining, the round or oval-shaped inclusions were red and surrounded by a clear halo (Fig. 6). Because of their cytoplasmic



TEXT-FIG. 1. Infectivity of MB-CVS and RE-CVS cell extract for tissue culture systems and mice.

localization and the percentage of cells showing their presence, these inclusions seemed to be the same as those demonstrated by FA.

Under a phase contrast microscope, no specific lesions were observed in any of the cellular organelles of cells of the RE-CVS series and the inclusions appeared as small, round or oval uniformly dense masses in the cytoplasm (see Fig. 7). Normal filamentous mitochondria were noted around the inclusions. The small fluorescent granules which were detected after staining with FA could not be seen after May-Grünwald–Giemsa staining, or observed under the phase contrast microscope.

After several passages, as the CVS became better adapted to growth in the RE cell system, it was possible to titrate the virus in the RE cells which were subsequently stained by FA. Text-fig. 1 shows the results of comparative titrations in RE and C-13 cells. As can be seen, the C-13 cells appeared to be equally susceptible to infection with MB-CVS and RE-CVS, and the titer of the RE-CVS in the RE cells was higher than that of the MB-CVS. These data show that less infectious virus is produced in the chronically infected cultures as compared to non-adapted virus grown in equal numbers of C-13 cells. Whereas the inclusions seen in the RE cells were small and medium-sized, the antigen in the infected C-13 cells appeared in the form of large, amorphous cytoplasmic inclusions, described elsewhere (1).

Identity and Properties of the RE-CVS.—Sonicated extracts of RE-CVS cells at the 42nd passage level were mixed with equal volumes of undiluted

TABLE I

Identification of CVS in the RE-CVS Culture*

Serum	Species	C-13 cells showing FA 7 days after exposure to virus-serum mixtures
		per cent
Antirabies	Human W	0
	Human F	0
	Horse P	0
Normal	Human	50
	Calf	75

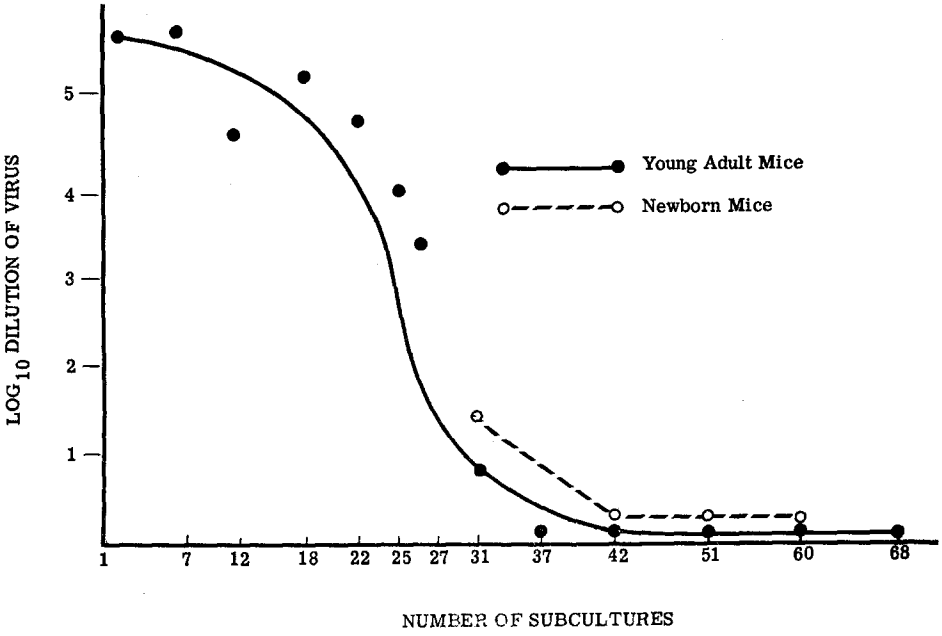
* Extract of sonicated cells mixed in equal parts with undiluted serum and the mixture incubated for 1 hour at 25°C.

normal and antirabies sera, as shown in Table I. These mixtures were incubated for 1 hour at 25°C and applied to monolayers of C-13 cells which were stained with FA after 7 days of incubation. The results indicate that the three samples of antirabies sera inhibited the formation of rabies antigen in C-13 cells (Table I).

Although the MB-CVS is virulent for adult mice, the results of intracerebral inoculation of mice with virus obtained from RE-CVS cultures indicated gradual loss of virulence for mice, even at an early passage level (Text-figs. 1 and 2). At the 30th cell transfer level, the mortality rate of adult mice injected with sonicated cell extracts obtained from RE-CVS was very low (Text-fig. 2); the same material was only slightly more virulent when injected into newborn mice. Finally, from the 42nd subculture or later, neither adult nor newborn mice died after intracerebral inoculation of RE-CVS cell extracts. Results of intracerebral challenge with MB-CVS indicated that mice injected

originally through 10^{-2} dilutions of the RE-CVS cell extract were protected. These extracts were still infectious for tissue culture systems at 10^{-3} or 10^{-4} dilutions (Text-fig. 2).

Newborn and young adult rabbits injected intracerebrally with 0.05 ml of a sonicated extract of RE-CVS cells at the 54th subculture level and observed for 5 weeks showed no signs of illness.



TEXT-FIG. 2. Loss of pathogenicity of readapted CVS for mice injected intracerebrally.

TABLE II

Antigenicity of RE-CVS Preparation Injected Intraperitoneally‡ into Mice*

Route of challenge	Results of challenge with MB-CVS			
	RE-CVS-immunized mice		Controls	
	LD_{50}	MR	LD_{50}	MR
Intracerebral	$10^{4.9}$		$10^{6.5}$	
Intramuscular		8/30		21/30

MR, mortality ratio.

* Extract of sonicated RE-CVS cells at the 45th subculture level which contained 10^4 $TCID_{50}$, as determined by titration on C-13.

‡ One intraperitoneal inoculation followed by challenge with MB-CVS 21 days later.

To test the antigenicity of the virus passaged in RE-CVS cells, sonicated cell extracts suspended in their own tissue culture medium were injected intraperitoneally into adult mice. These mice were challenged 21 days later with MB-CVS either by the intracerebral or intramuscular route (Table II). Immunized mice withstood the challenge better than the control mice (Table II), but the antigenicity of the RE-CVS, as determined by this mouse protection test was not very high.

Interference.—From the obtained data, it was apparent that virus antigen is carried in all RE-CVS cells and relatively small amounts of infectious virus are produced by these cells (Text-fig. 1). The RE-CVS system was, therefore,

TABLE III

Resistance of RE-CVS to Superinfection with Two Strains of Rabies Virus Pathogenic for Mice

Tissue culture	LD_{50} titer in mice inoculated with medium obtained from:		
	Non-infected cultures	PM-infected cultures	CVS-infected cultures
RE-CVS	< Undiluted	< Undiluted	< Undiluted
RE	0	$10^{3.5}$	$>10^{5.5}$

Titers of viruses grown in RE-CVS and RE cells were tested 6 days after infection with PM and CVS.

tested for susceptibility to superinfection with homologous and heterologous virus.

As shown in Table III, RE-CVS and RE cultures were exposed to infection with undiluted MB-CVS and RB-PM virus which remain pathogenic for mice after several passages in the RE tissue culture system (see above). Following 6 days' incubation, the culture media were titrated in mice injected intracerebrally. Whereas the LD_{50} titer of medium from the control RE culture infected with MB-CVS and RB-PM was $10^{3.5}$ and $> 10^{5.5}$, respectively, not a single animal died after inoculation with the undiluted medium obtained from RE-CVS cells superinfected with MB-CVS and RB-PM. Thus, growth of the RB-PM virus and MB-CVS was inhibited in the RE-CVS cells. Similar results were obtained when monolayers of RE-CVS and RE cultures were exposed to infection with heterologous VSV (Table IV) since RE-CVS inhibited multiplication of at least 7×10^3 or more PFU of VSV. These experiments demonstrate the capacity of RE-CVS cells to exclude replication not only of other closely related strains of rabies virus, but also unrelated VSV. In this respect, the RE-CVS system seems to differ from carrier cultures of polyoma L cells (11–13) and mumps in human conjunctiva cells (14) where interference was only observed against closely related viruses.

Repeated attempts to isolate an interferon-like substance from the RE-CVS cell system were unsuccessful.

Comparative Characteristics of the RE-CVS and RE Cell Lines.—Except for the intracytoplasmic inclusions which could be observed in RE-CVS cells under phase contrast or after staining, no differences could be detected between cells of the RE and RE-CVS series. Cells of both RE and RE-CVS cultures have an elongated epithelioid morphology; they exhibited the same growth rate when subcultured twice a week, and both demonstrated the contact inhibition phenomenon upon formation of a confluent monolayer.

The plating efficiency of the cells in the two systems was determined by plating 500 cells of each tissue culture line in 50 mm Petri dishes, incubating them

TABLE IV

Plaquing Efficiency of VSV in RE-CVS and RE Tissue Culture Systems

Tissue culture	No. of plaques of VSV per plate of culture exposed to virus dilutions (log_{10})				
	3	4	5	6	7
RE-CVS	0	0	0	0	0
RE	—Confluent—		37	7	0

VSV, vesicular stomatitis virus.

for 16 days, and then counting the number of colonies. The plating efficiency of the two cell lines was found to be very similar (Table V). There was no indication that the presence of rabies virus interfered with the viability of the RE-CVS cell system.

DNA- and RNA-Labeling Experiments.—The experiments dealing with the incorporation of labeled nucleic acid precursors failed to reveal any difference between the RE and RE-CVS cells. Thus, after 30 minutes of incubation with H_3-thymidine, the proportion of labeled nuclei was 14.8 per cent for the RE and 15.7 per cent for the RE-CVS cells. Given an approximate DNA synthesis time of 8 hours (21), it can be calculated that the generation time of both cell types is 50 hours. Even if H_3-thymidine was kept in the medium for as long as 7 hours, no cytoplasmic labeling occurred.

When H_3-uridine was used, the pattern of nuclear and cytoplasmic labeling in the two cell cultures was identical. The number of grains over the inclusions in the RE-CVS cells did not differ from other cytoplasmic zones. When the labeled precursor was present in the medium for 28 hours, no accumulation of RNase resistant RNA was observed anywhere in the cells. These findings indicate that the green staining of the inclusions by acridine orange previously reported (3) is not due either to the presence of DNA or of double stranded RNA.

Chromosomes of the Two Lines.—Although RE cultures are not strictly diploid, the karyotype of the RE-CVS cells in the 54th passage cells was examined to determine the effect of the virus infection on the chromosomes of its carrier cell.

Preliminary examination of chromosomes of 20 cells of the RE-CVS cell strain (91st passage) showed pseudodiploidy involving monosomy for one of the smallest chromosomes. Most of the counts of RE-CVS were near diploid, only 4 cells were exactly 44 (2n) and, of these cells, 3 were distinctly abnormal in chromosome morphology. However, examination of only 10 RE cells showed three instances of monosomy for one of the smallest chromosomes in three pseudodiploid cells, minute chromosomes, "true" chromatid breaks, and a tri-

TABLE V

Comparative Plating Efficiency of RE-CVS and Non-Infected RE Cultures

No. of cells plated	Culture	Experiment	No. of colonies	Plating efficiency
				per cent
500	RE-CVS	1	11	2.1
		2	14	2.8
	RE	1	16	3.2
		2	9	1.8

radiate configuration. The tetraploid level was increased from 7 per cent in the RE to 25 per cent in the RE-CVS cells. Because of these chromosomal aberrations in the non-infected RE culture, indicating instability, it was not possible to draw any conclusions as to the effect of the virus infection on the karyotype.

Effect of Antirabies Serum.—Several workers (11, 13, 14) have described how chronically infected cultures can be cured by transferring cells in the presence of specific antiviral serum. Similar studies were conducted with the RE-CVS culture after testing the effect of antirabies serum on the spread of extracellular virus.

Human antirabies serum (Human F) was inactivated for 30 minutes at 56°C and incorporated at a 20 per cent concentration into the tissue culture medium. For control purposes, normal medium and medium with added inactivated *normal* human serum were used. 2×10^5 RE-CVS cells were mixed in suspension with 2×10^6 RE cells and the mixture plated out on coverslips in Petri dishes (Table VI). All the plates were then incubated for 6 days, at which time the cells were stained by FA and May-Grünwald–Giemsa. The results, shown in Table VI, indicate that the presence of antirabies serum in the tissue culture medium prevented, to a large degree, the spread of extracellular virus from the RE-CVS cultures to the RE cells.

Following this experiment, an RE-CVS culture, starting from the 51st subculture passage, was maintained in immune serum medium (Human F) for nine consecutive cell transfers for a total of 53 days of cultivation. Even at the end of this period, 100 per cent of the RE-CVS cells showed the presence of inclusions stained by FA. Thus, it appears that while the antirabies serum effectively inhibited the spread of extracellular virus, its presence in culture medium could not interfere with the cell-to-cell transmission of the virus during mitosis.

TABLE VI

Prevention of Spread of Infective Virus from CVS-Infected RE Cells to Fresh RE Cells by Treatment with Antirabies Serum

Serum* in medium	Experiment	Cells 6 days after explanation of mixing‡ of RE-CVS and RE cultures showing:	
		FA	Inclusion bodies (May-Grünwald-Giemsa)
Antirabies§ (human)	1	20	20
	2	15	10
Normal (human)	1	100	70
Normal (calf)	1	100	75
	2	100	75

* At 20 per cent concentration.

‡ 2×10^5 cells of CVS-infected RE culture mixed with 2×10^6 cells of non-infected RE culture and explanted in cultures.

§ Human F serum inactivated for 30 minutes at 56°C.

Inactivated serum had to be used in these experiments since preliminary observations indicated that fresh antirabies serum was cytolytic for the RE-CVS cells. In order to investigate this point further, RE-CVS cells, either in suspension or in monolayer as shown in Table VII, were exposed to antirabies serum which was not cytolytic for RE cells (Human F). Complete cytolysis of the RE-CVS cells was observed after exposure to fresh antirabies serum whereas the same serum did not cause lysis of the same cells after inactivation for 30 minutes at 56°C. Addition of fresh hamster complement to the inactivated serum restored its cytolytic properties. There was no lysis of the RE cells exposed to the same fresh antirabies serum and, conversely, normal non-inactivated serum (without rabies antibodies) and complement had no effect on the RE-CVS cells.

Virus Release from Cells of the RE-CVS Cultures.—Since, as shown in Text-fig. 1, the extract of one million RE-CVS cells contained about 10^4 infectious

virus particles, it seemed improbable that all the cells of the culture appeared to be capable of releasing infectious virus at a given time even though virtually all of the cells showed the presence of rabies virus antigen. In the absence of a reliable plaque system for quantitative determination of virus, difficulties were encountered in deriving an assay for virus release from the individual RE-CVS cell. Finally, the following *ad hoc* technique was devised for this purpose. (See also Materials and Methods.)

TABLE VII

Cytolytic Effect of Antirabies Serum

Serum	Tfeatment of serum	Effect of serum on RE and RE-CVS cells			
		Cells in suspension		Cells in monolayer	
		RE-CVS	RE	RE-CVS	RE
Antirabies*	Non-inactivated	++++	0	++++	0
	Inactivated‡ plus complement§	+++	0	N.T.	N.T.
	Inactivated	0	0	0	0
Normal	Non-inactivated	0	0	0	0
Complement	Non-inactivated	0	0	N.T.	N.T.

0, no lysis.
++++, complete lysis with no recovery of viable cells.
+++, almost complete lysis but a few cells survived and gave origin to colonies.
N.T., not tested.
* Human F serum used in 1:4 dilution.
‡ Incubated for 30 minutes at 56°C.
§ 1:10 dilution of freshly collected hamster serum.

RE-CVS cells at the 84th subculture level were washed three times with PBS and diluted to a concentration of 1000 cells per 6 ml. Ten Petri dishes containing a monolayer of C-13 cells grown on two coverslips (11 x 22 mm) were seeded with 6 ml of RE-CVS cells per dish and incubated at 37°C. Eighteen hours later, one coverslip from each dish was removed and stained by FA in order to determine the number of dispersed RE-CVS cells which adhered to the C-13 monolayer on the coverslip. The other coverslip was removed after 6 days of incubation and also stained by FA. This time only the center of aggregations of 10 or more cells were counted. It was assumed that those cells were the C-13 cells infected by the virus released by the dispersed RE-CVS cells.

The results obtained with the coverslips removed after 18 hours and 6 days,

respectively, were matched for the same Petri dish (Table VIII). With the exception of coverslip 9, there was a fairly uniform count of dispersed RE-CVS cells adhering to the C-13 monolayer. Out of ten dishes showing the presence of RE-CVS cells, three failed to show fluorescing centers after 6 days of incubation, and in the remaining seven, the number of fluorescing aggregates of

TABLE VIII

Assay for Presence of Virus-Yielding Cells in RE-CVS

Time after seeding	No. of cells or cell aggregates showing FA										
	Coverslip No.										Average
	1	2	3	4	5	6	7	8	9	10	
18 hrs.*	26	35	46	39	42	31	33	44	12	39	34.7
6 days‡	0	3	0	1	3	1	4	2	0	1	1.5

* Dispersed RE-CVS cells.

‡ Aggregates of not less than 10 C-13 cells.

TABLE IX

Assays for Virus-Yielding Cells in RE-CVS in Presence of Antirabies Serum

Petri dish	Treatment	No. of cells or cell aggregates showing FA at time after seeding				
		18 hrs.		23 hrs.	6-days	
		No.*	Average	No.	No.‡	Average
1	*With* antirabies serum	103	76	0	2	4
2		92		2	6	
3		34		0	4	
4	*Without* antirabies	61	68	81	4	3
5	serum	74		53	2	

* Dispersed cells.

‡ Aggregates of not less than 10 cells.

presumably C-13 cells varied from one to four per dish. Taking into consideration the average values, about 4 to 5 per cent of the RE-CVS cells were releasing infectious rabies virus at a given time of cultivation.

To eliminate the possibility that aggregates of fluorescing cells, observed 6 days after the RE-CVS cells were seeded, represent the multiplying RE-CVS cells and not the fluorescing C-13 feeder layer cells, the experiment described above was repeated with a modification: C-13 monolayers were grown on three

coverslips per each of the five Petri dishes and, at 18 hours after seeding with RE-CVS cells when one coverslip was removed for staining, coverslips in three dishes were exposed to fresh antirabies serum (Human F) at a 1:4 concentration. Two Petri dishes were left without exposure to antirabies serum. Five hours later one coverslip from each of the five Petri dishes was removed for FA and the five dishes, still containing one coverslip each, were washed three times with PBS and reincubated until the 6th day after seeding when the remaining coverslips were stained with FA.

The results of the experiment, shown in Table IX, indicate that 5 hours after treatment with antirabies serum (23 hours after seeding) cells showing fluorescence at the 18th hour after seeding, were virtually eliminated. However, the number of colonies showing FA on the 6th day after seeding was essentially identical in cultures treated with antirabies serum and in those receiving no treatment at all. These results suggest that the fluorescence of cell aggregates observed on the 6th day after seeding with the RE-CVS cells was indeed that of the C-13 cells which became infected by the contact with RE-CVS cells during the 18 hour incubation period.

DISCUSSION

The results of the present study indicate that rabies virus can infect mammalian cells in culture and that it can propagate in the infected cells for a prolonged period of time, possibly indefinitely, without interfering with the mechanism of cellular replication. In this respect rabbit endothelial (RE) cells have the advantage over other cell systems infected with rabies virus (1, 2) because the virus remains in a true endosymbiotic state possibly in relation to every single cell in the culture. In systems such as human diploid cell strains and neonatal hamster kidney fibroblasts, the intracellular events following infection lead to the formation of large inclusion bodies which interfere with the mitotic process and ultimately cause degeneration of the culture (1, 2). No such phenomena were observed in the RE-CVS system. Cells, once infected, formed small to medium-sized inclusions, demonstrated either by FA or by histological means (3) and these inclusions did not seem to interfere with the mitotic process of the RE-CVS cells. Indeed, the rabies infected cultures (RE-CVS) has now been maintained for more than 93 cell transfers.

The term "endosymbiotic" has been used to describe the relationship between the rabies virus and the RE-CVS system since the more commonly used term "carrier" seemed to be reserved for a situation where only a fraction of the cell population at a given time is infected, and the virus constantly released in small amounts by these cells often infects other cells of the same culture. In the "carrier" system, the infected cells probably lyse continually, but their lysis escapes detection among the large fraction of non-infected, normally dividing cells (13).

There have been several reports of culture systems in which cells containing viral material undergo what appears to be a normal mitotic cycle (15–20). However, the RE-CVS system has been carried through more transfers than any other culture except for L cells infected with polyoma virus. In the latter case, the majority of the cells show the presence of viral antigen in the cytoplasm or in the RE-CVS system. However, in contrast to the RE-CVS cells, a small percentage of L cells infected with polyoma virus (a DNA virus) also show nuclear fluorescent staining. It has been postulated that these cells produce infectious virus (11) and lyse, and that, in other L cells, the presence of viral antigen in the cytoplasm could possibly be due to phagocytosed virus in the otherwise healthy cells.

Although all cells of the RE-CVS cultures showed the presence of viral antigen in the cytoplasm, it must still be determined why only 4 to 5 per cent release infectious virus at any given time.

Inquiry into this problem may be facilitated by finding methods which inhibit mitosis of RE-CVS cells and increase the virus yield.

Another important difference between the "endosymbiotic" infection and the "carrier" systems is that treatment with antiviral serum did not "cure" the virus infection in the RE-CVS cells during 9 cell transfers in the course of 53 days. In contrast, polyoma L cells were "cured" after exposure to antipolyoma serum for 81 days of cultivation (12).

The RE-CVS cells showed a resistance to infection with homologous as well as heterologous viruses in the absence of an interferon-like substance. Interferon could not be demonstrated in the polyoma L cell system (12) but in this case, in contrast to the RE-CVS cultures, interference was restricted to closely related viruses. Interference against closely related virus was also observed in another "carrier" system of human cell cultures chronically infected with mumps virus (14). The fact that all the cells of the RE-CVS system are infected by the rabies virus may in itself account for the resistance to infection by other viruses. This would eliminate the need for a transferable resistance factor produced by the infected cells and released into the culture medium.

The properties of the CVS became modified while the CVS was endosymbiotic with the RE cells. During the course of 40 passages, the virus lost not only its virulence for mice, but also, to a large extent, the ability to multiply in mice *in vivo* as indicated by the relatively poor antigenicity of the RE-CVS for mice. No pathogenicity for rabbits was observed after the passage of CVS virus in RE cultures.

Finally, it sould be pointed out that the sites at which the rabies virus persists in the body of an animal or man during the prolonged incubation period between exposure and illness are still unknown. Similarly, the mechanism through which the virus is capable of maintaining itself in such a latent state

remains a mystery. It is perhaps possible that a parallel may be found between the endosymbiotic relationship of the rabies virus to the RE-CVS cell and its prolonged persistence in cells of the whole organism without causing injury to the infected cells or to the host. What remains to be explained is the mechanism through which this endosymbiotic relationship is disrupted, possibly leading to the production of large quantities of virus which ultimately destroys the host.

SUMMARY

RE cultures infected with a fixed rabies virus were studied. The virus can propagate itself in these cells for an indefinite period of time without interfering with cell growth. The present study characterizes this truly endosymbiotic relationship.

Virus-specific antigen was detected in the cytoplasm of each cell by fluorescein-labeled antirabies serum but only 4 to 5 per cent of the cells released infectious virus. All cells undergoing division showed viral antigen throughout the mitotic process. Also, the growth rate, plating efficiency, and morphological characteristics of both the infected and control cultures were identical. No difference was detected between the RE and the RE-CVS cell populations by RNA and DNA labeling experiments, using H_3-thymidine, and H_3-uridine. Although antirabies serum effectively inhibited the spread of extracellular virus, it did not interfere with cell-to-cell transmission of the virus during mitosis, in the course of 9 cell transfers during 53 days. RE-CVS cells, when exposed to fresh antirabies serum, lysed completely but inactivated serum had no lytic effect. The addition of fresh hamster complement to the inactivated serum restored its cytolytic properties.

The serially passaged RE-CVS virus gradually became less virulent for mice and displayed a weak antigenicity in the mouse protection test.

Another feature of the RE-CVS cell system is its resistance to infection with homologous and heterologous viruses despite the apparent absence of an interferon-like substance.

The authors wish to express their sincere thanks to Dr. E. Wecker, Dr. V. Defendi, and other members of the staff at the Wistar Institute for their continuous help in the formation of these experiments and in the preparation of the manuscript, and to Mrs. Jean McClure and Mrs. Kathryn Wannemacker for excellent technical assistance. Dr. V. Defendi suggested and performed the experiments with the labeled nucleic acid precursors. Dr. P. Moorhead was responsible for the karyological analysis.

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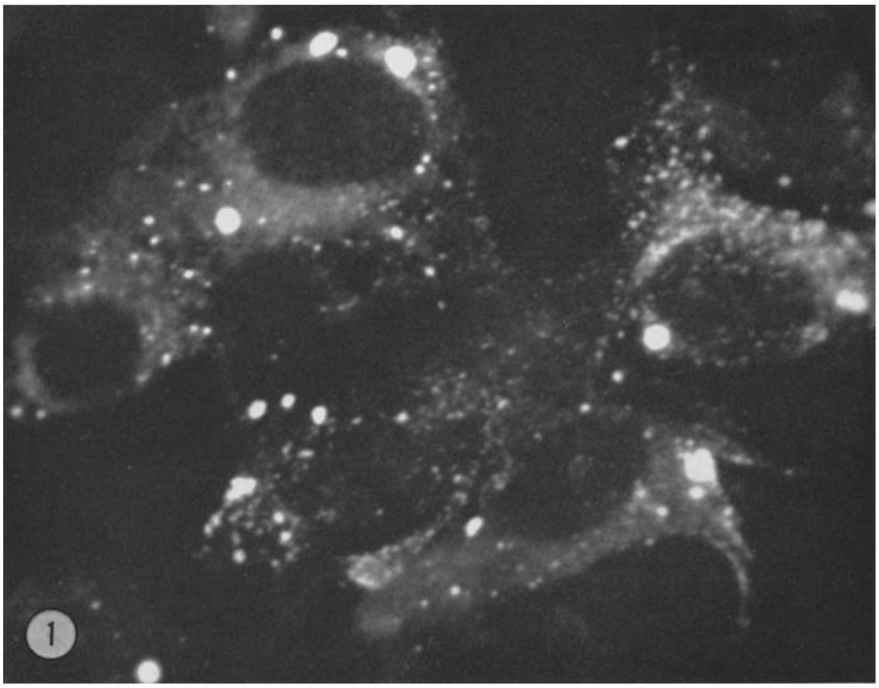
EXPLANATION OF PLATES

PLATE 98

FIG. 1. 62nd subculture of RE-CVS cells. Note the presence of bright fluorescent granules and small fluorescing inclusions in all cells. Immunofluorescent antibody staining. × 1200.



(Fernandes *et al.*: Animal viruses and host cells)

PLATE 99

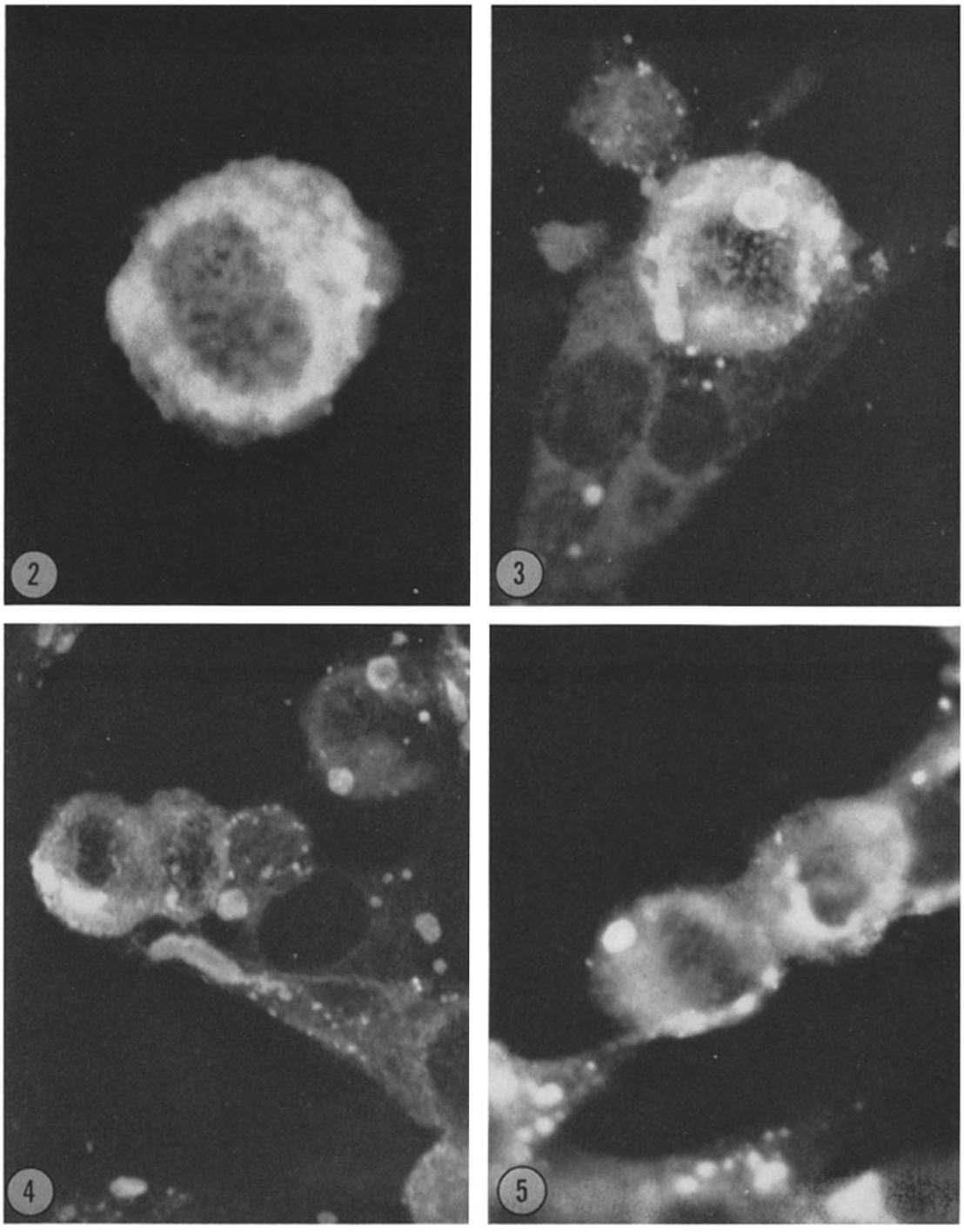
FIGS. 2 to 5. 53rd subculture of RE-CVS cells. Bright fluorescent granules in various stages of mitosis. Immunofluorescent antibody staining.

FIG. 2. Phophase. × 1400.

FIG. 3. Metaphase. × 1200.

FIG. 4. Anaphase. × 1200.

FIG. 5. Telophase. × 1200.

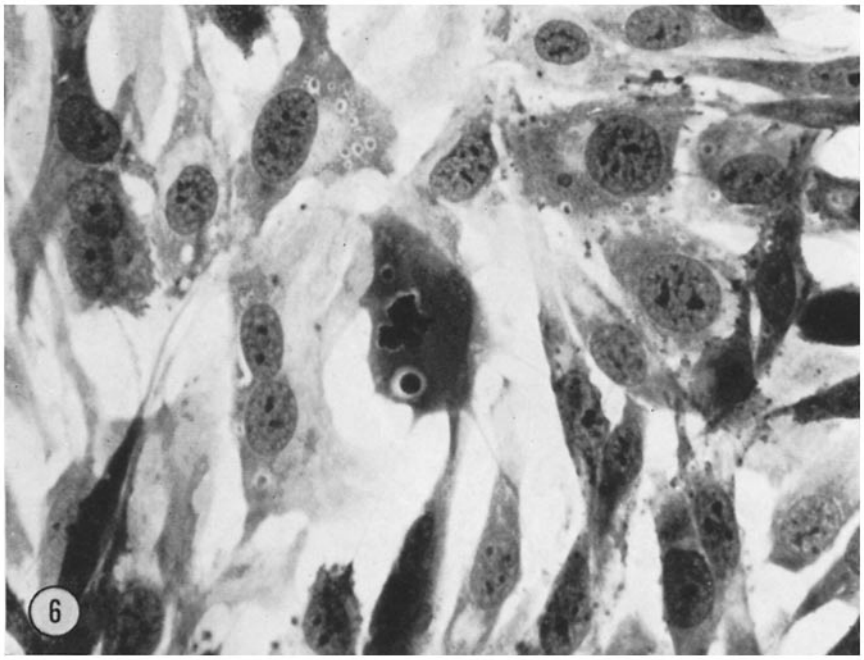


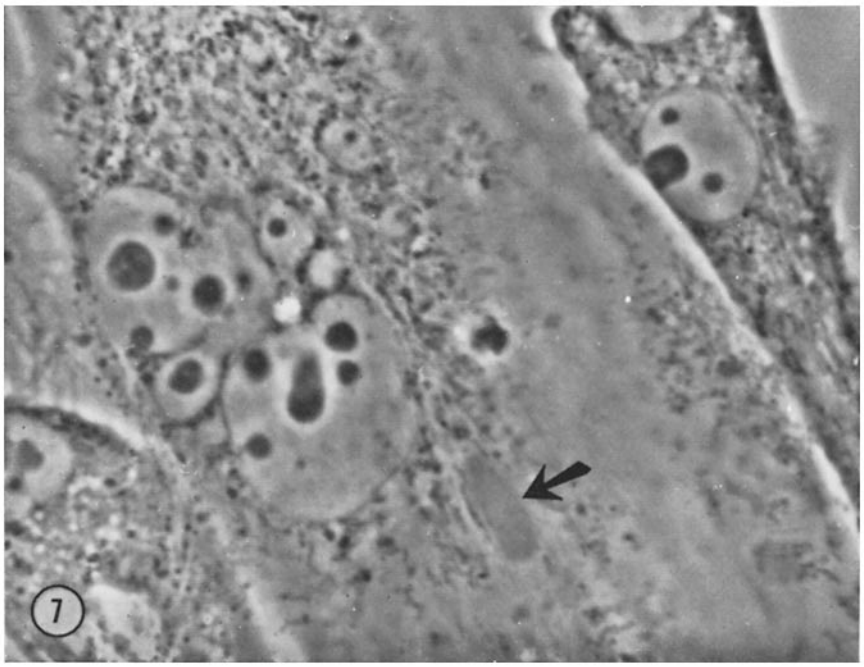
(Fernandes *et al.*: Animal viruses and host cells)

PLATE 100

FIG. 6. 87th subculture of RE-CVS cells. Note the presence of round or oval-shaped intracytoplasmic inclusions surrounded by a clear halo. May-Grünwald–Giemsa staining. × 740.

FIG. 7. 38th subculture of RE-CVS cells. Arrow indicates the oval, uniformly dense inclusions. Phase contrast microscopy. × 1200.





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