PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 163, 171–176 (1980)

Attempt to Demonstrate Virus Interference in Cell Cultures Persistently Infected with the Viruses of Kuru and Creutzfeldt–Jakob Disease (40742)[1]

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The viruses of kuru and Creutzfeldt–Jakob disease (CJD) have been shown to persist for long periods in cultures of brain cells derived from experimentally infected animals (1). Because these viruses replicate *in vitro* without recognizable cytopathic effect, the standard assay system consists of inoculating cultured cells into nonhuman primates with characteristic disease developing 1–2 years later (2, 3). Interference procedures have been used successfully in the past to detect and quantitate agents which are noncytopathic in certain cell cultures such as rubella virus (4). Consequently, in an effort to establish a more rapid and economical method of detection, an *in vitro* system based upon viral interference was tested. The present study describes our failure to demonstrate interference in cultures derived from explanted brain cells of chimpanzees experimentally infected with the viruses of kuru or CJD and Vero cells preinoculated with high titers of kuru or CJD virus using a large battery of animal viruses. Recently, however, we developed another *in vitro* method of detection based upon cell fusion by scrapie and CJD virus brain suspensions which eventually may serve as a quantitative tool (5).

Materials and methods. Cell lines. Control cell lines consisted of continuous lines of African green monkey kidney (Vero), HeLa cells, porcine kidney cells (PK-15), and primary human embryonic kidney (HEK). The HEK cells, obtained from Microbiological Associates, Bethesda, Maryland, were maintained with the basal medium of Eagle (BME) supplemented with 2–5% heat-inactivated fetal bovine serum and antibiotics. Vero and HeLa cells had undergone seven and nine passages, respectively, in this laboratory and were maintained with medium 199 or minimum essential medium (MEM) supplemented with serum and antibiotics as above. The PK-15 cells, obtained from Flow Laboratories, Rockville, Maryland, were maintained with MEM supplemented with 10% fetal bovine serum and antibiotics and in our hands had undergone three passages.

Chimpanzee brain cultures were prepared employing the explant technique previously described by Rogers *et al.* (2). Explants and subsequent passages were maintained with MEM supplemented with serum and antibiotics. Cell lines were derived from a chimpanzee (A-43) with experimental kuru, a chimpanzee (A-119) with CJD, and an uninoculated and apparently normal chimpanzee (C-8) obtained from another facility having had no contact with kuru or CJD. Chimpanzee A-119 was exhibiting clinical signs of CJD at sacrifice and chimpanzee A-43 was sacrificed during the early stages of kuru. Confirmation of kuru and CJD was made by demonstration of histopathological lesions indicative of the two diseases. Subsequent to the studies being reported here, inocula prepared from *in vitro* cultures of chimpanzee A-43 brain cells induced experimental kuru 18 months after intracerebral and peripheral inoculation into a chimpanzee. Inocula consisted of trypsinized cells of 27-day-old explants and a frozen and thawed suspension prepared from cultures at the third level which were derived from the original explants.

[1] This study was presented in part at the 76th Annual Meeting of the American Society for Microbiology, Atlantic City, New Jersey, May 13, 1976.

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0037-9727/80/020171-06$01.00/0

Attempts to isolate infectious agents other than kuru or CJD from the chimpanzee brains by inoculation into thioglycolate and Fletcher's media and onto sheep blood agar and cell culture monolayers were negative. Furthermore, reverse transcriptase assays failed to reveal the presence of reverse transcriptase-containing viruses in the cell cultures used in this study. Similarly, pig parvovirus, an agent known to infect a number of PK-15 sublines, was not detected in PK-15 cultures by hemagglutination, cocultivation, or immunofluorescent tests. Passage levels of the chimpanzee cell cultures used in this work were: A-43 passage 6, A-119 passages 6, 10, and 12, and C-8 passage 6.

Viruses. Challenge viruses were herpes simplex, vaccinia (DBS), measles (Enders), SV-5, parainfluenza 1 (HA-2), parainfluenza 3 (HA-1), reovirus (types 1, 2, and 3), vesicular stomatitis (Indiana), Tacaribe, Sindbis (Egypt), Bunyamwera, Japanese B encephalitis (M-543), St. Louis encephalitis (Hubbard), Langat (TP-21), Powassan (MacLean), and chimpanzee adenovirus (Pan 5). Pan is a laboratory designation for viruses which we have isolated from chimpanzees (2). Positive controls also were included consisting of rubella virus (Gilchrist) and Coxsackie A9 which served as the interfering virus and challenge virus, respectively. All challenge viruses were first passage suspensions grown in Vero, HeLa, PK-15, or HEK cells with the exception of measles which had undergone four passages in HEK cells.

Stock suspensions of brain tissue (20% w/v) were prepared in phosphate-buffered saline (PBS), pH 7.4, using mortars and pestles, clarified by low-speed centrifugation, and stored at −70°C. Suspensions were prepared from tissues obtained from normal chimpanzees (003039 and 003059), a chimpanzee killed in the intermediate stages of kuru (A-120), chimpanzees killed in the teminal stages of CJD (A-78 and A-82), and human patients that had died with kuru (Enage) or CJD (R.B.) and whose brain tissues when injected into chimpanzees had induced experimental disease. Final dilutions of brain suspensions were made with PBS, pH 7.4, prior to inoculation of cell cultures. Chimpanzees A-78 and A-82 were infected with the R.B. strain of CJD virus and chimpanzee A-120 received the Eiru strain of kuru. Chimpanzee and human brain suspensions were used in 10 and 1% concentrations, respectively, unless otherwise noted. Attempts to isolate other agents from these normal chimpanzee brains and from the human brains were negative. Foamy viruses were isolated from cell lines derived from brain tissues of chimpanzees A-82 and A-120, respectively, at later passage levels subsequent to the use of these lines in this study. These chimpanzee viruses did not appear in the passage level of chimpanzee brain cell cultures used in the interference tests.

Interference assays. Cell cultures employed in the interference experiments consisted of two groups: cultures derived from the brains of chimpanzees infected with kuru or CJD or from a normal chimpanzee brain, and Vero or PK-15 cell cultures preinoculated with high-titered brain suspensions from kuru or CJD cases or with normal brain suspensions.

Cultures derived from kuru- or CJD-infected or uninfected chimpanzee brains. Roller tubes of fully sheeted chimpanzee cell monolayers derived from brain explants of kuru (A-43), CJD (A-119), and normal (C-8) chimpanzees were challenged with 18 animal viruses. Serial 10-fold dilutions of the viruses in 0.1-ml volumes were inoculated into duplicate cultures and the cultures were periodically shaken for 1 hr at 35°C. Cultures were then incubated at 35°C and observed daily for 6–14 days for cytopathic changes. Infectivity titers ($TCID_{50}$) were calculated by the method of Reed and Muench (6).

Positive controls also were maintained. Confluent chimpanzee cell monolayers were inoculated with 0.1-ml volumes of rubella virus at a multiplicity of infection of 0.01. Rubella virus served as the interfering agent. Following incubation at 35°C for 7 days, duplicate cultures were challenged with 0.1-ml volumes of Coxsackie A9 virus at a multiplicity of infection of 0.001. After

incubation at 35°C for 7 days, the cultures were observed for the presence or absence of cytopathic changes.

Certain flaviviruses, namely, Japanese B encephalitis, St. Louis encephalitis, Langat, and Powassan, failed to produce CPE in the three chimpanzee cell lines. However, culture fluids from all three infected lines produced characteristic flavivirus CPE in PK-15 cells indicating that the chimpanzee cells were persistently infected with these agents. Infectious virus was detectable in culture fluids of the three lines inoculated with Japanese B encephalitis, Langat, and Powassan viruses for as long as 49 days after inoculation. St. Louis encephalitis virus was only detectable for 14 days postinoculation. Culture fluids were not tested for infectious virus beyond 49 days.

Furthermore, culture fluids from the three chimpanzee cell lines inoculated with each flavivirus were found to produce fatal encephalitis in newborn mice following intracerebral inoculation. Culture fluid specimens in these experiments were obtained from cultures inoculated 14 days earlier.

For interference tests, the chimpanzee cell lines were challenged with 10-fold dilutions of each flavivirus as above. On Day 5, 0.1 ml of culture medium from each dilution of the challenge virus titrations in the chimpanzee lines was transferred to cultures of PK-15 cells. These cultures were observed daily for 14 days for cytopathic changes and infectivity titers ($TCID_{50}$) were calculated.

Preinoculation of Vero or PK-15 cells with CJD or kuru or normal brain suspensions. Fully sheeted roller tube cultures of Vero cells or PK-15 cells were preinoculated in duplicate with 0.1-ml volumes of brain suspensions and shaken periodically for 1 hr at 35°C. The cultures were washed with fresh medium, fed, and incubated at 35°C for 7 days. Cultures were then challenged with 10 animal viruses in serial 10-fold dilutions as above. They were observed for 6–14 days and infectivity titers ($TCID_{50}$) were calculated by the method of Reed and Muench (6).

Results. Infectivity titers of the virus-infected chimpanzee brain and control cell lines are shown in Table I. There were no significant differences in titers among the CJD, kuru, and normal chimpanzee cell lines. Virus titers in the control lines were generally equal to or greater than those obtained in all chimpanzee lines studied. Only reovirus types 1 and 3 displayed lower titers in HEK cells than in the chimpanzee cell cultures. Chimpanzee cells were much less sensitive to adenovirus (Pan 5) infection than HEK cells. SV-5 virus did not display

TABLE I. INFECTIVITY TITERS ($TCID_{50}$/ml) OF CHALLENGE VIRUSES IN KURU- AND CJD-INFECTED AND UNINFECTED CHIMPANZEE CELL LINES AND CONTROL CELL LINES

Challenge virus	Control cell lines		Chimpanzee cell lines		
			Normal	Kuru	CJD
Adenovirus	HEK	7.0	4.0	3.0	4.5
Herpes simplex	HEK	6.5	5.0	6.5	5.5
Vaccinia	Vero	6.5	6.5	6.0	6.0
Measles	Vero	4.0	3.0	3.0	3.0
SV-5	Vero	7.5	0.0	0.0	0.0
Parainfluenza type 1	Vero	7.5	7.5	7.5	7.5
Parainfluenza type 3	Vero	7.0	6.0	6.5	6.0
Reovirus type 1	HEK	6.5	7.5	7.5	7.5
Reovirus type 2	HEK	7.0	7.0	7.0	7.5
Reovirus type 3	HEK	7.5	8.0	8.0	8.0
Vesicular stomatitis	Vero	9.0	8.5	8.0	8.5
Tacaribe	Vero	3.5	3.5	NT[a]	3.5
Sindbis	Vero	7.5	7.0	6.0	6.5
Bunyamwera	HeLa	8.0	7.0	6.0	6.5

[a] Not tested.

TABLE II. INFECTIVITY TITERS ($TCID_{50}$/ml) OF CHALLENGE VIRUSES TRANSFERRED FROM KURU- AND CJD-INFECTED AND UNINFECTED CHIMPANZEE CELL LINES AND ASSAYED IN PK-15 CELL LINE

Challenge virus	Chimpanzee cell lines[a]		
	Normal	Kuru	CJD
Japanese B encephalitis	4.5	3.5	3.5
St. Louis encephalitis	4.5	4.5	4.5
Langat	3.5	4.5	3.5
Powassan	4.5	4.5	4.5

[a] Since noncytopathic infections were established in the chimpanzee cell lines upon virus challenge, the dilutions of each titration were transferred from the chimpanzee cells to PK-15 cells, a cell line which manifests visible CPE, and the infectivity titers were subsequently calculated.

CPE in any of the chimpanzee lines. Noncytopathic infections of monkey cell cultures with SV-5 virus has been previously reported (7). Consequently, hemagglutination and hemadsorption properties of the SV-5-infected control and chimpanzee lines were tested. Positive hemadsorption and hemagglutination was obtained in the infected Vero cells through 10^{-6} dilution; however, no such activity was observed in the chimpanzee cell monolayers. In the positive controls, the chimpanzee lines preinoculated with rubella virus failed to show any evidence of cytopathology 7 days after challenge with Coxsackie A-9 virus indicating that virus interference indeed could be detected in this system. Furthermore, chimpanzee lines which were not preinfected with rubella virus displayed maximal Coxsackie CPE 7 days after inoculation.

The infectivity titers of challenge viruses, assayed in PK-15 cells following noncytopathic infections of CJD, kuru, and normal chimpanzee cell lines, are shown in Table II. There were no significant differences in the infectivity titers among the CJD, kuru, and normal chimpanzee cell lines.

Table III summarizes the infectivity titers of 10 challenge viruses in Vero or PK-15 cells preinoculated with brain suspensions from normal chimpanzees and from chimpanzees and humans affected with kuru or CJD. There were no significant differences in titers for a given virus in Vero or PK-15 cells preinoculated with different brain suspensions before challenge with the test virus; nor did these titers differ significantly from those obtained in untreated control cultures.

Discussion. Subacute spongiform encephalopathy is a generic term denoting the virus-induced slow infections of kuru and CJD of man, scrapie of sheep and goats, and mink encephalopathy (8). All affect

TABLE III. INFECTIVITY TITERS ($TCID_{50}$/ml) OF CHALLENGE VIRUSES IN VERO OR PK-15 CELLS PREINOCULATED WITH KURU- AND CJD-INFECTED HUMAN OR CHIMPANZEE OR NORMAL CHIMPANZEE BRAIN SUSPENSIONS

Challenge virus	Control cell lines		Cells preinoculated with brain suspensions from[a]				
			Normal chimp	Kuru chimp	CJD chimp	Kuru human	CJD human
Measles	Vero	4.0	NT[b]	4.5	5.5	5.5	4.5
SV-5	Vero	5.5	NT	6.0	5.5	5.5	6.5
Parainfluenza type 1	Vero	5.0	NT	5.0	5.0	5.0	4.5
Parainfluenza type 3	Vero	7.5	NT	6.5	7.5	7.0	7.0
Vesicular stomatitis	Vero	8.0	7.5	8.5	8.0	8.5	8.5
Tacaribe	Vero	3.5	3.5	3.5[c]	4.5[c]	3.5	4.0
Sindbis	Vero	8.0	7.6	7.5	6.5	7.5	8.0
Japanese B encephalitis	PK-15	3.5	3.5	4.5	4.5	4.5	3.5
Langat	PK-15	4.5	4.5	4.5	5.5	5.5	5.5
Powassan	PK-15	4.5	4.5	4.5	4.5	4.5	4.5

[a] Cultures preinoculated with 10% chimpanzee or 1% human brain suspensions.
[b] Not tested.
[c] Cultures preinoculated with 1% chimpanzee brain suspensions.

only the central nervous system; with histopathological lesions restricted to the gray matter of the brain and spinal cord, they present similar clinicopathologic pictures and are caused by unusual viruses which share many atypical biological and physical properties (8). The standard method for detecting and quantitating these agents has been based on the production of their respective diseases in inoculated subhuman primates or nonprimate hosts after incubation periods of months or years (2, 3), a procedure which is very time-consuming and costly. With the discovery that the viruses persisted in long-maintained primary brain cell cultures derived from affected patients or experimental animals, an attempt to demonstrate *in vitro* viral interference with other test viruses seemed reasonably justified.

Interference in which an interfering agent partially or completely prevents the replication of an unrelated or heterologous challenge virus and in which interferon is not involved was first described by Marcus and Carver (9, 10). Cell cultures infected with rubella virus, mouse leukemia virus, Sindbis, West Nile, or poliovirus were found to be resistant to infection with several strains of New Castle disease virus (NDV). Subsequent examples of heterologous interference have appeared and are reviewed by Fenner and his associates (11).

Albrecht has reported *in vivo* interference in scrapie-infected mice following challenge with Powassan virus (12); 20 to 100 times greater titers of Powassan virus were required to achieve infectivity in scrapie-infected mice than in control animals. A similar interference with the virus of louping ill has also been reported (13). We were unable, however, to demonstrate *in vitro* interference with Powassan virus and other flaviviruses in cell lines infected with the viruses of CJD and kuru. Similarly, we were unsuccessful in our attempts to demonstrate *in vitro* interference with agents of the flavivirus group in PK-15 or Vero cells preinoculated with human and chimpanzee brain suspensions containing the viruses of CJD and kuru. Dickinson *et al.* has shown that a strain of scrapie virus which is "slow" with respect to its rate of replication in the brain of a certain mouse genotype can completely interfere or block the replication of a "fast" strain injected later (14). It was suggested that during the process of replication the "slow" agent occupies replication sites which otherwise would be utilized by the "fast" agent.

Our inability to demonstrate *in vitro* interference in CJD- and kuru-infected cell cultures was not wholly unexpected considering the unusual nature of these agents. Both viruses appear to be completely nonimmunogenic and possess an inordinate degree of resistance to heating, formalin treatment, uv irradiation, and ionizing irradiation (15, 16). Such unusual biological and physiochemical properties set these agents apart from the majority of viruses infecting man (17). If they are also unconventional in their replicative cycle then blockage of classical replication sites or pathways might not occur thus permitting the unimpeded replication of conventional challenge viruses.

Recently, we described the induction of *in vitro* cell fusion by brain suspensions of scrapie and CJD virus (5). This important finding may not only enhance our understanding of the pathogenesis of the subacute spongiform virus encephalopathies but also may serve as a potential *in vitro* method to quickly and economically quantitate these agents.

Summary. Cell cultures persistently infected with kuru and CJD viruses were challenged with 18 different viruses in an effort to demonstrate *in vitro* interference. Cell cultures derived from the brains of a normal chimpanzee and a chimpanzee affected with kuru and CJD were challenged with the following viruses: herpes simplex, vaccinia, measles, SV-5, parainfluenza (types 1 and 3), reovirus (types 1, 2, and 3), vesicular stomatitis, Tacaribe, Sindbis, Bunyamwera, Japanese B encephalitis, St. Louis encephalitis, Langat, Powassan, and chimpanzee adenovirus (Pan 5). There were no significant differences in titers of any of these viruses, respectively, in the kuru, CJD, or normal chimpanzee cell lines. In addition, each of the 10 challenge viruses showed no significant differences in titers, respectively, in Vero or PK-15 cells

preinoculated with high-titered brain suspensions from man or chimpanzees with kuru or CJD or normal chimpanzee brain.

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Received July 24, 1978. P.S.E.B.M. 1980, Vol. 163.