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Attempts to Establish Cell Cultures Infected with the Viruses of Subacute Spongiform Encephalopathies (40974)[1]

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Abstract. Attempts to establish cell cultures infected with the viruses of scrapie, kuru, and Creutzfeldt–Jakob disease (CJD) were of limited success. Seven cultures of normal cell lines inoculated with brain suspensions containing kuru or CJD virus and nearly half (16/34) of the primary cell cultures derived from brains of humans and experimental animals with subacute spongiform encephalopathies were infectious. However, following prolonged maintenance or serial passages, cultures lost infectivity. Further, of 10 rapidly growing cell lines produced by SV40 transformation of cultures derived from eight scrapie-infected mice, one CJD-infected chimpanzee, and one kuru-infected chimpanzee, only one, a transformed scrapie mouse brain cell line, maintained infectivity. In all infectious cultures, a minimum of 10^4 cells were required to transmit 1 LD_{50} to an assay animal.

Studies in cell culture of the viruses of subacute spongiform encephalopathies—scrapie, transmissible mink encephalopathy (TME), kuru, and Creutzfeldt–Jakob disease (CJD)—have been of two principal types: (1) attempts to infect normal cell lines to establish an assay *in vitro;* and (2) attempts to derive cell cultures from infected tissues to investigate the infections at the cellular level. In the former type of study, inoculated cells have been examined for cytopathic effects (CPE) (1–3) or other changes, such as interference with the replication of challenge viruses (1, 4). In both types of study, a further objective has been to cultivate the filterable agents *in vitro* (2).

The purpose of this report is to summarize our experience with the viruses of spongiform encephalopathies in cell cultures, and to present recent findings on transformed cultures derived from tissues of infected animals.

Materials and methods. Virus isolation and propagation attempts. Normal cell lines—primary human embryo kidney cells (HEK), human embryo lung diploid fibroblasts (WI-38), human embryo brain diploid fibroblasts (FL-3000), and continuous African green monkey kidney cells (BSC-1)—purchased from commercial sources (5) were inoculated with brain suspensions containing the viruses of kuru and transmissible CJD (6). Suspensions were prepared as 5% homogenates. Cell cultures in roller tubes were drained, and 0.1 ml of virus suspension was inoculated. After 1 hr at 37°C 1 ml of maintenance medium was added to each culture without removing the inoculum. Treatments promoting cellular fusion or increasing permeability of cell membranes were not employed. Cells and supernatant fluids were transferred serially every 2 to 4 weeks and aliquots from selected passage levels were stored at −70°C until testing.

Cell cultures. Explant cultures from brains and other tissues of humans and animals with kuru or transmissible CJD were derived and maintained as described previously (7), except that in recent years Gro-BAF medium was replaced by Eagle's minimal essential medium containing 0.3% glutamine and 50 μg gentamicin per milliliter supplemented with 10 to 20% heat-inactivated (56°C for 1 hr) fetal calf serum for cell growth and 5% serum for maintenance. Most cultures were not passaged se-

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rially; instead they were maintained in their original flasks and the medium was changed at weekly intervals. Cells and fluids were collected periodically and were stored until assay. Trypan blue dye exclusion cell counts were performed in later years of the study.

Cell transformation. Simian virus 40 (SV40) was obtained from the American Type Culture Collection and was propagated and titrated in BSC-1 cells. Preparation of the virus pool and cellular transformation were performed according to the method of Todaro *et al.* (8). Brain cell monolayer cultures were inoculated with about 2×10^3 to 10^4 $TCID_{50}$ of SV40 per cell, and explants with 10^9 $TCID_{50}$ per 25-cm^2 plastic culture flask. Following inoculation, kuru- and CJD-infected chimpanzee brain cell cultures were maintained with growth medium containing 1% SV40-immune horse serum (Flow Laboratories).

The presence of SV40 tumor (T) antigen was demonstrated by the indirect immunofluorescence technique. Hamster antiserum to T antigen and fluorescein isothiocyanate-labeled goat anti-hamster immunoglobulin were kindly supplied by Dr. J. Gruber, Biological Carcinogenesis Branch, NCI. Successfully transformed cell lines were serially subcultured and then preserved in liquid nitrogen until assaying for infectivity.

Tests for infectivity. Various species of nonhuman primates known to be susceptible to the viruses of spongiform encephalopathies (9) were inoculated intracerebrally with 0.1 to 0.4 ml of undiluted or serial 10-fold dilutions of cells and culture fluids. In some cases, animals were also inoculated by the intravenous, intraperitoneal, and subcutaneous routes. SV40-transformed cell lines derived from brains of mice infected with the Chandler strain of scrapie virus (10) were assayed in outbred NIH Swiss albino mice as previously described (11). Only cultures with demonstrated infectivity and those on test for at least the mean incubation period plus two standard deviations for the particular animal species used for assay (9) were included in the analysis.

Results. Attempts to infect normal cell lines. Seven cultures of normal cell lines, which had been inoculated with brain suspensions containing kuru or CJD virus in high titer, were infectious up to 44 days later (Table I). Infectivity was not detectable in 17 other similarly treated cultures, the majority of which had undergone further subculturing (usually at 2- to 4-week intervals) and had been kept for longer periods following inoculation (Table I). None of the cultures exhibited CPE.

Attempts to establish cell lines by explanting infected tissues. Most cell cultures derived from nervous tissues of humans and primates with kuru and CJD grew slowly but were successfully maintained for extended periods. A total of 88 assays for infectivity were performed on cells and supernatant fluids of 34 cultures derived from infected tissues. Of these, 16 cultures, or 47%, retained the viruses of kuru and CJD. They consisted of 6 cultures derived from tissues of three patients (Ep, Ku, Um) and three animals (chimpanzees A-83, A-120, and A-43) with kuru (Table III), and 10 cultures derived from eight patients (T.At., M.Br., C.Ca., S.De., J.Do., D.Mu., T.Se., P.Wh.) and two animals (chimpanzee A-121, squirrel monkey SSC-5) with CJD (Table III). The maximum demonstrated periods of persistence of kuru virus in human and animal cultures were for 170 and 215 days, respectively (Table II); CJD virus persisted for 252 days in cultures of human origin and for 36 days in cultures of animal origin (Table III).

The incubation periods of CJD in two species of New World monkeys (squirrel and capuchin), and in chimpanzees infected with human cell cultures, ranged from 24 to 40 months, 11 to 35 months, and 14 to 27 months, respectively, and did not differ significantly from incubation times following inoculation of the same species with human brain homogenates (9). Similarly, two chimpanzees inoculated with cell cultures derived from kuru-infected chimpanzee brain tissue developed clinical disease in 14 months. In contrast, the incubation periods of kuru in three squirrel monkeys and one chimpanzee infected with human cell cultures and in four squirrel monkeys infected with cultures derived from brain

TABLE I. INFECTIVITY ASSAYS OF NORMAL CELL LINES INOCULATED WITH BRAIN SUSPENSIONS FROM KURU- AND CJD-INFECTED ANIMALS

Virus	Cell line[a]	Time in culture (days)	Number of passages	Infectivity assay[b]
Kuru (Se strain)				
Capuchin monkey AL-5,				
5% brain suspension	HEK	21	1	+ (1/1)
	HEK	44	2	+ (1/1)
	HEK	85	4	− (0/1)
	HEK	145	6	− (0/1)
	BSC-1	21	1	+ (1/1)
	BSC-1	44	2	− (0/1)
	BSC-1	72	3	− (0/1)
	BSC-1	85	4	− (0/1)
	BSC-1	145	6	− (0/1)
	FL-3000	21	1	+ (1/1)
	FL-3000	72	3	− (0/1)
	FL-3000	85	4	− (0/1)
	FL-3000	145	6	− (0/1)
	WI-38	21	1	+ (1/1)
	WI-38	44	2	− (0/1)
	WI-38	72	3	− (0/1)
	WI-38	85	4	− (0/1)
	WI-38	145	6	− (0/1)
CJD (R Re strain)				
Chimpanzee A-156,				
5% brain suspension	HEK	22	1	+ (1/1)
	HEK	40	2	− (0/1)
	BSC-1	22	1	+ (1/1)
	FL-3000	22	1	− (0/1)
	FL-3000	40	2	− (0/1)
	WI-38	40	2	− (0/1)

[a] HEK = primary human embryo kidney cells, BSC-1 = continuous African green monkey kidney cells, FL-3000 = human embryo brain diploid fibroblasts, WI-38 = human embryo lung diploid fibroblasts.

[b] Capuchin and squirrel monkeys were inoculated intracerebrally (and in some cases by other routes as well) with 0.2- or 0.3-ml aliquots of undiluted cells and culture fluids which had been frozen and thawed once. + = spongiform encephalopathy produced in the animals. Only animals on test for at least the mean incubation period plus two standard deviations (capuchin monkey, 60 months; squirrel monkey, 36 months) and not having signs of neurological disease are included as negative (−). Most animals have now been on test for more than 6.5 years.

tissues of two kuru-infected chimpanzees (chimpanzees A-83 and A-120) were 31 to 59 months, 57 months, and 38 to 45 months, respectively, and were significantly longer than the incubation times in the same species after inoculation with brain homogenates.

The remaining 18 cultures, 9 each from kuru- and CJD-infected tissues, were not infectious when tested in primates (Tables II and III). Most of these cultures had been maintained for periods exceeding 1 year (6/9 kuru cultures) or had been passaged three or more times (5/9 CJD cultures). These noninfectious brain cell cultures did not appear morphologically different from cultures with demonstrated infectivity.

Undiluted and serial 10-fold dilutions of cells or of cells and supernatant fluids of 9 cultures derived from kuru-infected tissues and 18 cultures derived from CJD-infected tissues were tested for infectivity. Although precise cell counts of all inocula were not available and the total number of assays per dilution was small, those cultures counted generally yielded about 10^6 cells suspended in 10 ml. Since no infectivity was demonstrated in any of 8 kuru and 7 CJD cultures

TABLE II. INFECTIVITY ASSAYS OF CELL CULTURES DERIVED FROM TISSUES OF PATIENTS AND ANIMALS WITH KURU

Virus strain	Source of culture[a]		Time in culture[b] (days)	Passages[c]	Inoculum[d]	Infectivity assays[e]
	Patient/animal	Tissue(s)				
Ei	Chimpanzee A-83	Brain, ?region	188	1	SC,10^{-1}	+ (1/1)
			215	1	SC,10^{0}	+ (1/1)
	Chimpanzee A-88	Brain, pooled regions	238	1	SC,10^{0}	− (0/1)
	Chimpanzee A-120	Brain, ?region	70	1	SC,10^{0}	+ (1/1)
			70	1	SC,10^{1}	+ (1/1)
En	Chimpanzee A-2	Brain, pooled regions	842–994	1	SC,10^{0}	− (0/1)
Ep	EP	Cerebellum	170	1	S,10^{0}	+ (1/2)
			164–383	1	S,10^{0}	− (0/1)
Ka	Chimpanzee A-7	Brain, pooled regions	990–1047	1	SC,10^{0}	− (0/1)
		Cervical cord	1117–1143	1	SC,10^{0}	− (0/1)
Ki	Chimpanzee A-41	Brain, pooled regions	459	1	S,10^{0}	− (0/2)
Ko	KO	Brain, pooled regions	141–176	1	S,10^{0}	− (0/2)
		Cerebellum	102–141	1	S,10^{0}	− (0/1)
Ku	KU	Spinal cord	112–133	1	S,10^{0}	+ (1/1)
			345–357	1	S,10^{0}	− (0/2)
Mo	Chimpanzee A-5	Brain, pooled regions	854–958	1	SC,10^{0}	− (0/1)
		Thalamus, frontal	712–789	1	SC,10^{0}	− (0/1)
Se	Chimpanzee A-43	Brain, pooled regions	26–27	1–3	SC,10^{0}	+ (1/1)
			538	32	SC,10^{0}	− (0/2)
Um	UM	Cerebellum	124–184	1	S,10^{0}	+ (1/1)

[a] Specific source of explant culture is indicated when known, otherwise the organ is described as in the original protocols.

[b] Where several cultures are combined, the shortest and longest times in culture are indicated.

[c] When several cultures are combined, the lowest and highest passage levels are indicated; the original explant culture is designated passage 1.

[d] The preparation used in the infectivity assay: S = culture fluids with floating debris; C = sedimented cells resuspended in a small volume of culture fluid; 10^{0} = undiluted; 10^{-1} = 10% dilution. Higher dilutions are omitted.

[e] Primates known to be susceptible to kuru were inoculated intracerebrally with 0.1 to 0.4 ml cells and/or culture fluids. Some animals were inoculated by other routes as well. The infectivity assay was considered to be negative (−) if the inoculated animal had been on test for at least the mean incubation plus two standard deviations for the test species (chimpanzee, 60 months; squirrel monkey, 36 months; spider monkey, 48 months; capuchin monkey, 60 months). Most negative animals have been on test for 8 years. Results are expressed as positive animals in the numerator and total number of animals in the denominator.

diluted 100-fold or more, it is estimated that at least 10^4 cells were required to transmit disease. Even using undiluted cell suspensions (10^5/ml) only 5/13 kuru and 10/33 CJD cultures tested were found to be infectious.

Attempts to establish transformed cell lines. Two cell lines, one derived from the brain biopsy specimen of a man with transmissible CJD and the other from the brain of a mouse with scrapie, developed altered morphology and rapid growth, and appeared to be spontaneously transformed. A retrovirus, later identified as related or identical to the Mason–Pfizer monkey virus (12), was recovered from the former cell line (13). The mouse brain cell line contained RNA-dependent DNA polymerase activity (M. A. Lewis and G. J. Nemo, unpublished data). Neither cell line retained infectivity when tested at various passage levels.

Eight cell cultures derived from the

TABLE III. INFECTIVITY ASSAYS OF CELL CULTURES DERIVED FROM TISSUES OF PATIENTS AND ANIMALS WITH CREUTZFELDT–JAKOB DISEASE

Virus strain	Source of culture[a]		Time in culture[b] (days)	Passages[c]	Inoculum[d]	Infectivity assays[c]
	Patient/animal	Tissue(s)				
T At	T AT	Frontal cortex	10	1	SC,10^0	+ (2/2)
M Br	M BR	Frontal cortex	42	1	SC,10^0	+ (1/1)
C Ca	C CA	Brain, ?region	36	1	S,10^0	− (0/2)
			36	1	C,10^0	+ (1/2)
			90	1	S,10^0	+ (1/1)
J Co	J CO	Frontal cortex	109	1	SC,10^0	− (0/1)
			109	1	SC,10^{-1}	− (0/1)
R Cu	Squirrel SSC-22	Cerebellum	82	1	SC,10^0	− (0/1)
S De	S DE	Frontal cortex	66	1	SC,10^0	− (0/2)
			66	1	SC,10^{-1}	+ (1/1)
			113	3	SC,10^0	− (0/2)
J Do	J DO	Brain, ?region	252–255	1	SC,10^0	+ (1/1)
	(+W NE)	Brain, ?region	111–255	1	SC,10^0	+ (1/1)
S Gr	S GR	Brain (biopsy)[f]	109	4	SC,10^0	− (0/1)
			204	24	SC,10^0	− (0/2)
		Brain (autopsy)	118	3	SC,10^0	− (0/1)
E Hi	E HI	Brain, ?region	63	2	C,10^0	− (0/1)
			89	3	C,10^0	− (0/2)
			139	5	C,10^0	− (0/1)
D Li	D LI	Brain, ?region	38	1	SC,10^0	− (0/1)
			38	1	SC,10^{-1}	− (0/1)
D Mu	D MU	Brain, ?region	237–300	1–3	SC,10^0	+ (1/1)
E Ne	E NE	Cerebellum	70	3	SC,10^0	− (0/1)
R Po	R PO	Brain, ?region	79	1	SC,10^0	− (0/1)
			104	2	SC,10^0	− (0/1)
A Re	A RE	Brain, ?region	50	1	SC,10^0	− (0/2)
			50	1	SC,10^{-1}	− (0/1)
			139	4	SC,10^0	− (0/2)
			139	4	SC,10^{-1}	− (0/1)
R Re	Chimpanzee A-121	Brain, pooled regions	36–42	1–3	SC,10^0	+ (1/1)
T Se	T SE (+A EL)	Brain, pooled regions + spleen, adrenal, other viscera	150–296	1	SC,10^0	+ (1/1)
A Ta	Squirrel SSC-5	Cerebellum	29	1	SC,10^0	+ (1/1)
P Wh	P WH	Frontal cortex	31	1	S,10^0	+ (1/2)
			31	1	S,10^{-1}	+ (1/1)

[a–d] See footnotes *a–d* in Table II.

[c] Primates known to be susceptible to Creutzfeldt–Jakob disease were inoculated intracerebrally with 0.1 to 0.4 ml cells and/or culture fluids. Some animals were inoculated by other routes as well. Only animals on test for at least the mean incubation period plus two standard deviations (chimpanzee, 60 months; squirrel monkey, 36 months; capuchin monkey, 60 months) without developing signs of neurological disease were considered to be negative (−). Most animals have now been on test for more than 7 years. Results are expressed as positive animals in the numerator and the total number of animals inoculated in the denominator.

[f] This brain culture was spontaneously transformed.

brains of mice affected with scrapie and two cultures derived from the brains of chimpanzees with kuru and CJD were easily transformed with SV40. (The chimpanzee cell cultures, unlike those of human cells (14), did not undergo lytic infection with SV40.) Cell transformation was characterized by the acquisition of rapid growth, altered morphology, and T antigen, and the loss of contact inhibition in some cases. Low-titered scrapie infectivity (1 LD_{50} per 10^4 cells) was demonstrated in one SV40-transformed scrapie mouse brain cell line assayed at the 12th passage level, which represented a 10^{-8} dilution of the original explanted culture, suggesting that replication of scrapie virus had occurred. However, when both unselected and multiple

cloned cultures of this line were assayed in mice at the 30th to 35th passage levels, scrapie activity was not detected. The remaining seven SV40-transformed mouse lines contained no scrapie infectivity even at low passage levels.

The two SV40-transformed chimpanzee brain cell lines and the two original cell lines from which they were derived were serially subcultured until they had undergone final dilutions of 10^{-15}, a dilution adequate to eliminate all of the original infectious agent in the absence of replication. Neither the untransformed nor the transformed cell cultures have produced disease in squirrel monkeys after nearly 4 years of observation.

Discussion. Several investigators have previously reported that persistently infected cell cultures could be easily derived from brains of mice with scrapie (3, 15, 16) and mink with TME (17). In our accumulated experience with multiple strains of the spongiform encephalopathy group of viruses in tissue culture, however, it was striking that most cultures were infectious for only limited periods. Although the viruses of kuru and CJD persisted for weeks to several months in some primary cell cultures derived from infected tissues (18), they were not detected in primary cultures maintained longer than about 9 months or in cultures that were serially passaged more than once. It would appear that either the viruses or the infected cells replicated only slowly if at all, that only a few cells in the original culture were infected, and that those cells were eventually greatly outnumbered by uninfected cells.

When infectivity titers were compared with the number of cells in individual cultures, it appeared that a minimum of 10^4 cells were required to transmit 1 LD_{50} of kuru or CJD virus. Similar results were obtained with an SV40-transformed mouse brain cell line, in which scrapie virus persisted and possible replicated. These results are consistent with previously reported findings (17, 19). Marsh and Hanson demonstrated that, although the corneal epithelium of hamsters dying from TME contained the virus in high titer (about 1 LD_{50} per cell), the infectivity of corneal epithelial cells was reduced dramatically (to 1 LD_{50} per 10^4 cells) after just one passage in tissue culture (19).

Subtle abnormalities such as multinucleation (3, 20), vacuolation (21, 22), and transformation (23) in cell cultures derived from brains with spongiform encephalopathies have occasionally been reported; however, these abnormalities were rarely observed in our cultures and cannot serve to distinguish between cultures from infected brains and those from normal or uninfected brains.

Whether the process of transformation or the transforming agent interfered with replication of the viruses of spongiform encephalopathies remains uncertain, but only one of 12 spontaneously or experimentally transformed cell cultures derived from kuru-, CJD-, and scrapie-infected tissue retained infectivity. Moreover, unlike the SMB line of Clarke and Haig which has maintained appreciable infectivity (about 1 LD_{50} per 100 cells) during many years of subculturing (24, 25), the sole infectious SV40-transformed scrapie mouse brain cell line appeared to lose infectivity on serial passage. Although it is possible to compute the approximate total number of cells containing one LD_{50}, the actual number of infected cells required to transmit scrapie, kuru, or CJD to an animal host from such a culture has not been defined.

Using lysolecithin-treated membrane fractions of scrapie-infected brain tissue as the inoculum, Clarke and Millson successfully infected mouse L cells with scrapie virus (26). The infectivity titers of two such infected cell lines were low (approximately 1 LD_{50} per 3000 cells), but multiplication of virus was demonstrated. Other attempts to infect cell cultures derived from normal sheep (1, 3), goats (1), and mice (1, 2) by exposure to suspensions of scrapie virus have been unsuccessful, as were those reported here. Attempts to produce infected mouse L cells by selection of fused hybrid colonies induced by scrapie-infected suspensions (20) have thus far been unsuccessful (C. Kidson *et al.*, unpublished data), but further attempts using lysolecithin and other membrane-disrupting agents should be made.

Although multinucleation has been observed in normal cell cultures exposed to high concentrations of infected brain tissue suspensions (3, 20), this finding is not necessarily consistent with, nor restricted to, suspensions of brain tissues from subjects with spongiform encephalopathies (27). Cultures so treated were often normal in appearance, and had normal susceptibility to infection with challenge viruses (1, 4). Moreover, while some virus-treated cultures were initially infectious, they rapidly lost infectivity on serial passage, indicating that there had been little or no virus replication (1, 2). Our data are consistent with those findings. The viruses of kuru and CJD did not persist in normal lines of human or nonhuman primate origin for more than two passages or for periods longer than 44 days.

Whether derived by explanting tissues of affected patients or experimental animals, or by infecting normal cell lines with infectious brain suspensions, all cell cultures have much lower titers of virus than do infected brain tissues *in vivo*. Since cell cultures usually lose all detectable infectivity with time and routine manipulation, they cannot replace tissues from infected animals as sources of virus for studies of viral structure. However, if used soon after explanting or carefully selected by assay in animals, infected cells in culture may prove useful in investigations of the biology of spongiform encephalopathies, and further efforts to prepare cultures of higher infectivity should be made.

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