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Comparison of Primary Skunk Brain and Kidney and Raccoon Kidney Cells with Established Cell Lines for Isolation and Propagation of Street Rabies Virus

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Cell cultures prepared from skunk kidney, raccoon kidney, and skunk brain were compared with CER, murine neuroblastoma (C1300, clone NA), baby hamster kidney (BHK-21, S-13), and dog kidney (MDCK) cell lines for virus isolation and propagation of street and fixed rabies virus. The skunk brain cells were suitable for efficient replication of all the virus isolates. They were comparable to CER and murine neuroblastoma cells for virus isolation and propagation. None of the other cell cultures was satisfactory. Further work is under way to refine the skunk brain cell cultures.

Fixed rabies viruses were first maintained in vitro in fragments of brain and other nerve tissue by Noguchi in 1913 (2). Field isolates of rabies virus have never been found to efficiently infect most cell lines (5). Murine neuroblastoma (NA) and CER cell lines have been shown to be suitable for primary isolation of field isolates (4). Since skunks and raccoons are natural reservoirs of rabies, we hypothesized that cell cultures developed from the brain or kidney of either species would be comparable or superior to NA and CER cells for primary isolation. This study was undertaken to test this hypothesis.

Baby skunks (*Mephitis mephitis*) were obtained from the field and killed by instant decapitation. The preparation of the skunk brain cells was routine (1) except that 0.125% trypsin in Hanks balanced salt solution (pH 7.8) was used instead of 0.25% trypsin. Flasks were seeded with 10^6 cells per ml. The cells were incubated in a closed system in Glasgow minimum essential medium (G-MEM) supplemented with 15% fetal bovine serum, 70 μg of gentamicin per ml, and 2.0 mM L-glutamine (Sigma Chemical Co., St. Louis, Mo.) or in Dulbecco minimum essential medium (D-MEM) supplemented as G-MEM was, but grown in 5% carbon dioxide. Kidney tissue was secured from the same baby skunk (BSK). Raccoon kidney tissue was secured from adult raccoons trapped from the wild (RKC). Preparation of these cells was essentially the same as for skunk brain cells. Only cells free from cytopathological changes and infectious agents were used for virus isolation and propagation. Electron microscopic studies were conducted using cells in 3 to 5 passages which were lysed and examined for the presence of agents (6). The preexisting cell lines used for comparative purposes were NA, CER, baby hamster kidney (BHK-21, S-13), and dog kidney (MDCK) cells.

For passage 2, eight-chambered Lab-Tek slides (Lab-Tek Division, Miles Laboratories Inc., Naperville, Ill.) or 25-cm^2 flasks were seeded with 2×10^5 cells in 200 μl of growth medium (G-MEM supplemented with 10% fetal bovine serum, 70 μg of gentamicin per ml, and 2.0 mM L-glutamine) and held for 24 to 72 h at 37°C until a monolayer was formed. The viruses were in original brain tissue from infected dogs, skunks, CVS-infected mouse brain, ERA-infected pig kidney cells, experimentally infected goats, and uninfected goat brain used as a control.

To inoculate the eight-chambered slides, the medium was aspirated, and 20% suspensions of the immunofluorescence-positive brain tissue in 0.75% bovine serum albumin or 10-fold dilutions of the tissue in growth medium (for titration) were added to the monolayer. After absorption for 90 min, the cell sheet was washed twice with Dulbecco phosphate-buffered saline, pH 7.5 (PBS), and overlaid with 400 μl of maintenance medium, viz., G-MEM supplemented with 2% fetal bovine serum, 2.0 mM L-glutamine, and 70 μg of gentamicin per ml and containing 55 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Sigma Chemical Co.) and 56 mM sodium hydroxide. The slides were incubated for 3 days at 35°C. The cells were then washed with PBS, fixed with cold acetone at −20°C, and stained with fluorescein isothiocyanate-labeled

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TABLE 1. Virus isolation attempts using established cell lines and primary skunk brain cells, determined by immunofluorescence

Virus isolate	Fluorescence[a] in following cell cultures:						
	CER	NA	BHK	MDCK	BSK	SBC	RKC
BA dog	+	+	+	+	+	+	+
MO dog	+	+	−	−	−	+	−
G-44 goat	+	+	+	+	+	+	+
G-45 goat	+	+	−	−	−	+	−
Sk-a skunk	+	+	−	−	−	+	−
Sk-b skunk	+	+	+	+	+	+	+
SK-c skunk	+	+	+	+	−	+	−
CVS mouse	4+	4+	2+	2+	3+	4+	2+
ERA porcine cell culture	4+	4+	2+	2+	2+	4+	2+
G-51 goat, normal	−	−	−	−	−	−	−

[a] −, No fluorescing cells were observed in any fields; +, fluorescing cells in up to 5 fields; 2+, fluorescing cells in 6 to 10 fields; 3+, fluorescing cells in 11 to 15 fields; 4+, fluorescing cells in 16 to 20 fields. In all cases, 20 microscopic fields were examined.

immunoglobulin. For titration, a 50% tissue culture infective dose was calculated by the method of Reed and Muench (3).

To inoculate flasks, the growth medium was removed, and each monolayer was washed twice with PBS and treated with 50 μg of DEAE-dextran per ml in growth medium at room temperature for 10 min. A 20% suspension of immunofluorescence-positive brain in 0.75% buffered bovine serum albumin (500 μl) or ERA vaccine reconstituted with 2.0 ml of growth medium (500 μl) was put on the cell monolayer. After 90 min of absorption at 37°C, the monolayer was washed twice with PBS and overlaid with 5.0 ml of G-MEM containing 2.0% fetal bovine serum and 70 μg of gentamicin per ml. The flasks were incubated at 35°C for 4 days. Neuroblastoma cells were grown in D-MEM, supplemented as G-MEM was, and incubated at 37°C in the presence of 5% carbon dioxide. After day 4 of incubation, the medium was removed and stored at −70°C until used for titration. Infected cells were scraped from the monolayer in the flasks with a rubber policeman, dispersed, washed with PBS, and resuspended in a small volume of PBS. Smears were made on glass slides, air dried, fixed in cold acetone for 30 min, and stained with fluorescein isothiocyanate-labeled globulin for examination by immunofluorescence microscopy. At least 500 cells were counted, and the percentage of fluorescing cells was calculated.

Examination was carried out using a Leitz Ortholux II microscope with epi-illumination and an HBO 100 illuminator. The filtering system consisted of KG-1 heat absorption, BG-23 red suppression, 2-KP 490 (=KP500) excitation, and K495 suppression filters. Interpretation of results was based on the presence of intracellular apple-green fluorescence in discrete dots or

TABLE 2. Increase in titer of rabies virus from original brain tissue to serial passage 4 in various cell cultures[a]

Virus isolate	Titer in original brain tissue	Titer at passage 4 in following cells:				
		NA	BHK	BSK	SBC	RKC
BA dog	1.7	5.1	4.5	4.5	4.5	3.9
MO dog	<1.7	4.2	ND[b]	2.7	3.5	ND
G-44 goat	1.7	4.7	5.1	2.7	4.0	3.2
G-45 goat	<1.7	4.5	ND	ND	5.1	1.9
Sk-a skunk	3.2	5.4	4.2	3.6	5.2	2.7
Sk-b skunk	2.2	4.5	3.2	3.2	4.9	3.5
Sk-c skunk	3.2	6.0	4.5	3.8	5.7	3.0
CVS mouse	6.2	7.1	6.5	6.7	7.1	6.5
ERA porcine cell culture	4.5	6.7	5.5	5.8	6.5	6.0
G-51 goat, normal	<1.7	<1.0	<1.0	<1.0	<1.0	<1.0

[a] The titers are expressed as log 50% tissue culture infective doses per 0.1 ml in CER cells. MDCK cells not done.

[b] ND, Not determined.

TABLE 3. Percentages of fluorescing cells during primary propagation (counted 3 days postinfection) and at passage 4.

Virus isolate	% at 3 days postinfection/% at passage 4 in following cell cultures:						
	CER	NA	BHK	BSK	MDCK	SBC	RKC
BA dog	16/95	14/100	1/76	0.5/81	1.3/79	19/94	2/71
MO dog	6/92	5/100	0/44	0/35	0/ND[a]	3/89	0/22
G-44 goat	9/91	17/100	1/57	2/45	0.5/65	12/100	2.5/85
G-45 goat	6/89	13/97	0/22	0/73	0/59	8/90	0/19
Sk-a skunk	6/79	9/100	0/45	0/49	0/35	6.5/92	0/31
Sk-b skunk	5/90	11/100	0.5/55	0.5/60	1/56	4/91	1/49
Sk-c skunk	10/87	23/95	0.5/61	0.5/43	0.5/ND	7/63	1/56
CVS mouse	20/100	42/100	7/100	12/100	6/100	21/100	11/89
ERA porcine kidney cells	41/100	81/100	80/100	37/100	28/93	73/100	35/100

[a] ND, Not determined.

conglomerates and the absence of these in the inhibition control slides.

All the virus isolates replicated efficiently in the CER, NA, and SBC cell lines. Fixed viruses (mouse CVS and ERA vaccine viruses) replicated efficiently in all cell lines (Table 1). BSK and RKC cells did not support efficient growth of field strains of rabies virus on primary isolation. Only three of the seven field isolates could be identified as having infected BSK or RKC. The results obtained with these cell cultures were similar to those obtained with BHK and MDCK cells.

Each virus isolate had an increase in titer with serial passage, regardless of the cell system used. With many isolates, titers obtained at passage 4 with infected NA and SBC were higher than titers obtained at the same passage level with infected BHK, BSK, MDCK, and RKC (Table 2). The number of fluorescing cells increased with subcultivation of the infected cells; when the percentage of fluorescing cells with a cell system was compared with the percentage of fluorescing cells within other cell systems, significant differences were obtained (Table 3). Differences in the percentage of fluorescing cells between CER and BSK, CER and BHK, NA and BSK, NA and BHK, NA and RKC, SBC and BSK, SBC and BHK, and SBC and RKC were significant ($P < 0.5$). There were no statistically significant differences in the percentage of fluorescing cells between CER and NA, CER and SBC, or NA and SBC. Similar results were obtained with passage levels 1 and 4.

These results confirm the previous findings that NA and CER cell lines could be used for isolation of field strains of rabies virus (4) and also indicate the suitability of the use of skunk brain cells for primary isolation. The three field isolates from the skunks did not grow efficiently in the skunk kidney cells but did in the skunk brain cells, suggesting that the type of cell (nervous or kidney cells) was a more important factor than was adaptation to the species as a whole. The SBC culture was prepared from whole brains of baby skunks. A mixed population of cells of the brain was obtained which supported efficient replication of field isolates of rabies virus without prior adaptation. The quantity of the virus particles in the inoculum, the type and the susceptibility of the cells, and previous adaptation of the virus to a cell culture or other host system are among the important factors that might affect the efficient replication of rabies virus in a cell culture system. We are currently examining selected cell type cultures from the mixed brain cell population.

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