

SUSCEPTIBILITY OF VARIOUS CELL LINES TO RABIES VIRUS

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Abstract: Twelve established cell lines including 7 mouse-derived cell lines and primary chick embryo fibroblast cells were examined for their susceptibility to rabies virus. The results indicated that, in addition to CER cells and BHK-21 cells, murine neuroblastoma cells and human neuroblastoma cells (SYM cells) are highly permissive to infection with both fixed and field rabies strains and suggested the usefulness of these neuronal cell lines in various immunologic aspects of rabies studies in which histocompatibility requirements play a significant role.

INTRODUCTION

Rabies virus has been successfully propagated in many tissue culture systems in the past (Kissling, 1958; Wiktor *et al.*, 1964; Kondo, 1965; Diamond, 1967; Hummeler *et al.*, 1967; Sedwick and Wiktor, 1967; Wiktor *et al.*, 1969). These include primary cell cultures such as hamster kidney cells and chick embryo fibroblast cells, and human diploid cells which have been used mainly for the purpose of vaccine production (Kissling, 1958; Wiktor *et al.*, 1964; Kondo, 1965; Wiktor *et al.*, 1969; Grandien, 1977). Of established cell lines, BHK-21 cells and their sublines have been used by several investigators for the production of high yields of virus (Sokol *et al.*, 1968, 1969; Schneider *et al.*, 1971; Cox *et al.*, 1977; Kawai and Matsumoto, 1977). CER cells, recently established by Dr. Motohashi, Japan, have also been demonstrated to be highly permissive to rabies virus infection and to have a potential for isolation of field strains of rabies virus (Smith *et al.*, 1977, 1978).

One aspect of the search for susceptible cells in tissue culture systems is to develop appropriate tissue culture cells for use in *in vitro* tests of immunological responsiveness to rabies in laboratory animals and in human. In these sorts of studies, it has been demonstrated that a histocompatible system (H-2 in the mouse) is required for the full expression of the interaction between immune lymphocytes and the virus-infected target cells (Doherty and Zinkervagel, 1975). The present study was initiated to examine the susceptibility of mouse-derived cell lines to rabies virus, with the ultimate goal of studying the immunological responses to the virus in mice. In addition, some commonly used cell types derived from other animals were simultaneously tested for permissiveness to the virus.

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MATERIALS AND METHODS

Virus: Three strains of rabies virus were used. The HEP-Flury strain of rabies virus was supplied by Dr. Kondo, National Institute of Health, Tokyo, Japan, and used at passage levels of 106 to 111 in chick embryo fibroblast cells. HEP-Flury of 2nd to 3rd passage level in CER cells was used in some experiments. The mouse brain-adapted CVS strain was used as a 10 per cent infected mouse brain suspension at the 56th passage level. In some experiments, the CVS strain, plaque-cloned in Vero cells (Buckley and Tignor, 1975) obtained from Yale Arbovirus Research Unit (YARU), Yale University, was used after one additional passage in murine neuroblastoma cells. The 1088 rabies strain, originally isolated from a woodchuck by the Center for Disease Control, U.S.A., was obtained from YARU and used at the 3rd passage level in suckling mouse brain.

Cells and culture media: Seven mouse-derived cell lines and 6 cell cultures derived from other animals (5 continuous lines and primary chick embryo fibroblasts) were tested for their susceptibility to rabies virus. Growth media for these cells were shown in Table 1. After virus infection, the percentage of heat-inactivated fetal calf serum was reduced to 2 or 5 per cent.

Table 1 Cell lines tested for susceptibility to rabies virus

Mouse-derived		Other animal-derived	
Cell	Growth medium	Cell	Growth medium
L	Eagle MEM+10% FCS ^{a)}	Vero	Eagle MEM+10% FCS
L 1210	RPMI 1640 +20% FCS	BSC-1	Eagle MEM+10% FCS
L 5178Y	RPMI 1640 +20% FCS	BHK-21	Eagle MEM-Hanks salts +10% FCS
Neuroblastoma (N-18)	Dulbecco's modified Eagle MEM+10% FCS	CER	Eagle MEM+10% FCS
SR-CDF1-DBT ^{b)} (glioma)	Eagle MEM+10% FCS	SYM ^{c)} (Human neuro- blastoma)	1/2 Eagle MEM +1/2 RPMI 1640 +20% FCS
Mastocytoma (P-815)	Eagle MEM+10% FCS	CEF ^{d)}	Eagle MEM-Hanks salts +10% FCS
Ehrlich ascites tumor	Eagle MEM+10% FCS		

a) Heat-inactivated fetal calf serum. All culture media contained antibiotics.

b) Established by Dr. T. Kumanishi (1967), Tokyo University, Japan.

c) Established by Dr. M. Sekiguchi (personal communication), Tokyo University, Japan.

d) Primary chick embryo fibroblast cells.

Parameters of susceptibility: Infection of the cells with virus was carried out in the presence of 50 $\mu\text{g/ml}$ of DEAE-dextran for 1 h at 37 C. Infected cultures were then refed with maintenance media or overlay media and incubated at 37 C. At least 2 tests using the following parameters were performed to examine permissiveness.

a) *Fluorescent antibody staining.* The cells capable of adhering to glass were cultured in 4-chamber Lab-Tek chamber slides (Lab-Tek Products, Miles Laboratories, Naperville, Ill.) and infected at a multiplicity of infection (MOI) of approximately 0.1 focus forming unit (FFU)/cell and incubated in a 5% CO₂ incubator. Other cells not capable of adhering to glass were packed by low speed centrifugation and infected at an MOI of 0.1 FFU/cell. The cells were then transferred to test tubes at appropriate cell densities and incubated.

On days 1, 2, 3 and 4 after infection, these cells were examined for cytoplasmic fluorescent antigens using anti-rabies virus FITC-labelled goat serum. The percentage of cells containing specific cytoplasmic fluorescent antigens was determined by examining at least 300 cells. The amount of cytoplasmic fluorescent antigens in the cells was estimated as large, intermediate and minute, respectively, depending on the relative size and distribution of inclusions in the cells.

b) *Cytopathic effect.* Monolayers in culture flasks and suspension cultures in test tubes, both infected with virus at a MOI of 0.1 FFU/cell, were observed for visible cytopathic effect under an inverted microscope for 8 days.

c) *Plaque formation.* The ability of some cells to support plaque formation was also tested in certain cell lines. The procedures were essentially the same as those described by Yoshino *et al.* (1966) except that calf serum and agar in the overlay were replaced by heat-inactivated fetal calf serum and 2% Sephadex G-200 (Schneider, 1973), respectively. Infected cultures were observed for plaques for 8 days.

d) *Hemadsorption-negative (HAD⁻) plaque formation.* This was done as described by Makino and Mifune (1975) for dengue virus assay.

e) *Persistent infection.* To examine if the percentage of fluorescent antigen-positive cells increased during subcultures, the cultures infected with virus at a MOI of 0.1 FFU/cell were refed with growth medium and subcultured 4 times at an interval of 5 to 7 days with a split ratio of 1 to 4. At the time of subculture, the percentage of fluorescent antigen-positive cells in a culture was determined as described before. The cultures which contained no cells with cytoplasmic fluorescent antigens at the 2nd and the 4th subculture levels were considered to be "cured".

f) *Virus yield.* Monolayers of susceptible cells in culture flasks were infected with virus at a MOI of 0.1 FFU/cell and incubated for 4 days. The cultures were frozen and thawed 3 times and the supernatant was assayed for infectivity after low speed centrifugation. Infectivity of the virus was assayed in CER cells by the fluorescent focus formation method using 4-chamber Lab-Tek chamber slides as described by Smith *et al.* (1977).

RESULTS

Of seven mouse-derived cell lines tested, neuroblastoma cells were most sensitive to all strains of rabies virus tested. By fluorescent antibody staining, almost all of the N18 cells contained large, widely spread fluorescent antigens by day 4 after infection even with a field rabies strain (1088), and persistent infection could be readily established. In contrast, SR-CDF1-DBT cells and Ehrlich ascites tumor cells were sensitive only to the tissue culture-passaged, attenuated HEP-Flury strain

of rabies virus. Susceptibility of other cell lines derived from the mouse was found to be very low (Table 2).

Table 2 Susceptibility of mouse-derived cell lines to rabies virus as determined by fluorescent antibody staining, CPE induction and ability to support virus growth during subculture

Cell		Virus strain		
		1088	CVS (mouse brain- passaged)	HEP-Flury
L	FA ^{a)}	0-2 ^{b)} (M) ^{c)}	0-2 (M)	0-2 (M)
	CPE ^{d)}	—	—	—
	Persistent infection	cure	cure	cure
L 1210	FA	20-30 (M)	20-30 (M)	15-35 (M)
	CPE	—	—	—
	Persistent infection	cure	cure	cure
L 5178 Y	FA	10-20 (M)	10-20 (M)	10-20 (M)
	CPE	—	—	—
Neuroblastoma (N-18)	FA	95-100 (L) ^{e)}	95-100 (L)	95-100 (L)
	CPE	—	±	±
	Persistent infection	yes	yes	yes
SR-CDF1-DBT (glioma)	FA	N.D. ^{g)}	0-2 (M)	80-90 (I) ^{f)}
	CPE	—	—	—
Mastocytoma (P-815)	FA	0-0.1 (M)	0-0.1 (M)	0-0.1 (M)
	CPE	—	—	—
Ehrlich ascites tumor	FA	N.D.	0-2 (M)	80-95 (L)
	CPE	—	—	—

a) Fluorescent antibody staining.

b) Per cent of fluorescent antigen containing cells on day 4 after infection in 3 experiments.

c) Minute, pinpoint like antigen.

d) Cytopathic effect.

e) Large, widely spread antigen masses in the cells.

f) Intermediate antigen.

g) Not done.

Cell lines derived from other animals appeared, in general, to be more permissive to rabies virus infection than were the mouse-derived cells, and it was difficult to compare the susceptibility to rabies virus among these cell lines. CER and SYM cells, however, had the capacity to replicate the 1088 strain to almost the same degree as the fixed rabies strains as determined by fluorescent antibody staining, and the infection of CER cells could be successfully maintained in serial subcultures (Table 3). Vero and BSC-1 cells were sensitive only to fixed rabies strains (CVS and HEP-Flury strains) by fluorescent antibody staining but produced lower yields of the virus as

Table 3 Susceptibility of non-mouse-derived cell lines to rabies virus as determined by fluorescent antibody staining, CPE induction, plaque and HAD⁻ plaque formation and ability to support virus growth during subculture

Cell		Virus strain		
		1088	CVS (mouse brain-passaged)	HEP-Flury
Vero	FA ^{a)}	5-10 ^{b)} (M) ^{c)}	80-90 (I) ^{d)}	80-90 (I)
	CPE	—	+	+
	Plaque	—	+	—
	Persistent infection	cure	yes	yes
BSC-1	FA	5-10 (M)	50-75 (I)	50-75 (I)
	CPE	—	—	—
	HAD ⁻ plaque	—	+	+
BHK-21	FA	20-25 (I)	95-100 (L) ^{e)}	95-100 (L)
	CPE	—	+	±
	Persistent infection	yes	yes	yes
CER	FA	50-100 (I)	95-100 (L)	95-100 (L)
	CPE	—	+	—
	Plaque	—	+	—
	HAD ⁻ plaque	—	+	+
	Persistent infection	yes	yes	yes
SYM (Human neuroblastoma)	FA	65-75 (I)	95-100 (L)	95-100 (L)
	CPE	—	±	±
CEF ^{f)}	FA		30-50 (I)	95-100 (L)
	CPE	N.D. ^{g)}	—	±
	Plaque		—	+

a) Fluorescent antibody staining.

b) Per cent of fluorescent antigen containing cells on day 4 after infection in 3 experiments.

c) Minute, pinpoint like antigen.

d) Intermediate antigen.

e) Large, widely spread antigen masses in the cells.

f) Primary chick embryo fibroblast cells.

g) Not done.

compared with those of CER and BHK-21 cells (Table 4). BSC-1 cells were shown to be useful for the infectivity assay of fixed strains of rabies virus. Vero cells and CER cells permitted plaque formation by the CVS strain of rabies virus.

Virus yields from a limited number of susceptible cell lines are shown in Table 4. In general, tissue culture-passaged rabies virus grew to higher titers than did mouse brain-passaged rabies virus. Among these cell lines, murine neuroblastoma cells and CER cells supported replication of tissue culture-passaged CVS rabies to the highest titers. The highest yields of HEP-Flury virus were obtained in CER cells and CEF cells.

Table 4 Rabies virus yields from susceptible cell lines^{a)}

Cell	Virus strain		
	CVS (mouse brain- passaged)	CVS (plaque-purified murine neuroblastoma-passaged)	HEP-Flury
Murine neuroblastoma (N-18)	5.3 ^{b)}	8.0	5.9
Vero	3.6	6.4	5.8
BSC-1	3.2	N.D. ^{c)}	5.0
BHK-21	5.0	7.0	6.3
SYM (Human neuroblastoma)	5.5	7.2	N.D.
CER	5.7	7.7	7.3
CEF ^{d)}	N.D.	N.D.	7.3

a) Monolayers in culture flasks were infected with virus at an MOI of 0.1 FFU/cell and incubated at 37 C for 4 days. The cultures were frozen and thawed 3 times and supernatant fluid was assayed for infectivity after low speed centrifugation.

b) Log₁₀ FFU/ml.

c) Not done.

d) Primary chick embryo fibroblast cells.

DISCUSSION

Murine neuroblastoma cells, which were derived from an A strain mouse and which express some neuronal characteristics in culture, were demonstrated to have the greatest susceptibility to rabies of the mouse-derived cell lines tested in this study. Since these cells permit expression of antigen on the surface after infection with both fixed and street rabies virus, they have been recently chosen as target cells by us (Mifune and Tignor, in press) and others (Wiktor *et al.*, 1977) in the *in vitro* studies of immunological responses of rabies in mice. In addition, these cells have potential for isolation of field rabies strains (Smith *et al.*, 1978) and provide a system in which to examine the interaction between field rabies strains and neuronal cells.

The susceptibility of L cells and mastocytoma cells derived from C₃H mouse and DBA mouse, respectively, was shown to be very low. The high susceptibility of CER cells to rabies virus including field strains was first described by Smith *et al.* (1977). In this study, further and comparative observations were made with some other cell lines regarding their susceptibility to the virus. The results showed that, in addition to CER and BHK-21 cells, SYM cells derived from a human neuroblastoma are highly susceptible to both fixed and field rabies strains and might be a useful cell line for the immunological studies of human rabies which might require a histocompatible system in the assays.

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狂犬病ウイルスに対する種々細胞株の感受性

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7株のマウス由来組織培養細胞株を含む12の細胞株と初代鶏胎児細胞について、狂犬病ウイルスに対する感受性を検討した結果、これまで報告のあった CER 細胞ならびに BHK-21 細胞の他に、マウス神経芽細胞腫およびヒト神経芽細胞腫 (SYM 細胞) 由来の細胞が、狂犬病ウイルスの固定毒ならびに街上毒に高い感受性を示し、マウスおよびヒトにおける狂犬病の研究、特に、組織適合抗原の適合性が重要な免疫学的研究に、これらの細胞が非常に有用であることが示唆された。

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