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Comparison of Sensitivity of BHK-21 and Murine Neuroblastoma Cells in the Isolation of a Street Strain Rabies Virus

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The sensitivities of BHK-21 (C-13) and murine neuroblastoma (C-1300; clone NA) cells for the isolation of small quantities of a street strain rabies virus were compared. Suspensions of brain from mice sacrificed prior to the onset of clinical signs of rabies were used to stimulate weakly positive diagnostic specimens. The results of cell culture isolation were compared with those of the direct fluorescent-antibody test and virus isolation in weanling mice. Neuroblastoma cells were more sensitive to the street strain rabies virus than were BHK-21 cells. Neuroblastoma cell virus isolation, the mouse inoculation test, and the fluorescent-antibody test all showed comparable sensitivity.

Since the decision to withhold rabies postexposure vaccination following an animal bite is frequently based upon the results of laboratory diagnosis, the procedures used must be fast, specific, and very sensitive. The fluorescent-antibody test (FAT) has proven to be such a procedure when performed by an experienced laboratory with high-quality reagents (5, 19). False-negative FAT results are not common but can occur (3). Because of the high medical significance of these examination results, virus isolation is commonly used as a back-up procedure to the FAT in rabies diagnosis. Since 1935 (17) the intracerebral inoculation of mice (mouse inoculation test [MIT]) has served to confirm microscopic examination results for rabies diagnosis.

The use of tissue cultures for the growth of rabies virus was first described in 1913 (9); see Wiktor and Clark for a review of this subject (18). Until recently it had been widely assumed that street virus strains adapted slowly to growth in vitro. Therefore, this procedure had not been used as a routine diagnostic procedure. In 1978, Smith et al. (14) demonstrated that street virus strains could be readily isolated in CER and murine neuroblastoma cells. In 1980, Rudd et al. (12) showed that BHK-21 cells sensitized with DEAE-dextran (6, 8) were equal to the MIT in sensitivity to the street virus strains tested. In titrations, neuroblastoma cells appeared to be more sensitive to street rabies virus than did BHK-21 cells (16).

The purpose of this study was to compare the sensitivity of virus isolation in BHK-21 and murine neuroblastoma cells. Test inocula were prepared to contain the smallest amount of antigen detectable by the FAT, thereby stimulating specimens that most require prompt confirmation by a sensitive virus isolation procedure. The results of the in vitro isolation were compared with those of the FAT and MIT for each sample.

MATERIALS AND METHODS

Cell cultures and media. BHK-21 cells (C-13) (ATTC CCL 10) (American Type Culture Collection, Rockville, Md.) were used at passages 70 to 95. Mouse neuroblastoma cells (C-1300) (clone NA) (10), a gift from T. J. Wiktor, Wistar Institute, Philadelphia, Pa., were used at passages 30 to 50. Both cell lines were grown in Eagle minimum essential medium supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, 2.0 mM glutamine, 2.0% sodium bicarbonate, 200 IU of penicillin, and 0.4 mg of streptomycin per ml (Eagle growth medium). Cell pools were prepared by trypsinization of 75 to 95% confluent tissue culture flasks. The trypsinized cells were suspended in room-temperature Eagle growth medium and cooled rapidly in an ice water slurry. To prevent the clumping of cells, we constantly stirred the suspension with a pipette during cooling. The cell concentration was adjusted to 6.0×10^5 cells per ml with cold Eagle growth medium. A stock suspension of DEAE-dextran (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in Eagle minimum essential medium (10 mg/ml) was added to the cold cell suspension, resulting in a final concentration of 50.0 μg of DEAE-dextran per ml of cell suspension. A new sensitized cell suspension was prepared each day a test was performed.

Virus and test inocula. The virus isolate was from a naturally infected red fox submitted to the New York State rabies diagnostic laboratory. A 10% fox brain suspension was prepared in Eagle growth medium. To produce test suspensions containing very small amounts of virus, we inoculated 20 Nya:NYLAR mice (10 to 12 g) intracerebrally with the 10% fox brain suspension and sacrificed them 130 h postinoculation, prior to the onset of clinical signs of rabies. Ten percent suspensions in Eagle growth medium and microscope slide slip smears were prepared from homogenized brain of individual mice. The suspensions were centrifuged under refrigeration for 30 min at $3,000 \times g$ and the supernatant fluids were used as the test inocula.

FAT. The FAT was performed by the technique of Dean and Abelseth (2) with the following modifications. Tissue culture test slides and slip smears of mouse brain were air dried and fixed in acetone for 1 h at −20°C. Slides were stained for 45 min with a commercially available fluorescein-tagged monoclonal antibody conjugate (pH 7.6) (Centocor, Inc., Malvern, Pa.) and then washed by immersion in phosphate-buffered saline (pH 7.6) for 5 min.

Tissue culture slides were examined at a magnification of 240×, and slip smears of mouse brain were examined at a magnification of 600×. FAT results for mouse brain slides were graded on a negative to 4+ scale corresponding to the presence and quantity of antigen detected. All slides were independently evaluated by two microscopists.

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TABLE 1. Comparison of the FAT and viral isolation procedures with 20 mouse brains containing small amounts of rabies virus antigen

Sample	FAT result	MIT result (no. positive/ no. inoculated)	RTCIT result (% cells positive) with:	
			BHK-21 cells	Neuroblastoma cells
1	+1	2/5	−	+ (1)
2	+1	3/5	−	+ (1)
3	+1	0/5	−	+ (1)
4	+1	1/5	−	−
5	+1	4/5	−	+ (3)
6	+1	4/5	−	+ (2)
7	+1	3/5	−	+ (2)
8	+1	5/5	+ (3)	+ (25)
9	+1	4/5	−	+ (4)
10	+1	3/5	−	+ (1)
11	±	0/5	−	+ (One cell)
12	+1	5/5	+ (1)	+ (30)
13	+1	4/5	−	+ (2)
14	+1	2/5	−	+ (1)
15	+1	3/5	−	+ (2)
16	+1	5/5	+ (2)	+ (20)
17	+1	4/5	−	+ (5)
18	+1	4/5	−	+ (3)
19	+1	2/5	−	+ (1)
20	+1	2/5	−	+ (1)

MIT. Five weanling (10 to 12 g) Nya:NYLAR mice were inoculated intracerebrally with 0.03 ml of test suspension and checked daily for 30 days. At the first sign of a clinical infection, the mice were asphyxiated with CO_2, and slip smears of brain tissue were examined by the FAT. The suspension was positive if one or more of the mice contracted rabies. Mice were housed in negative-pressure biohazard containment isolators which were exhausted outdoors through high-efficiency filters.

RTCIT. The in vitro isolation of virus (rabies tissue culture infection test [RTCIT]) was performed in eight-well Lab-Tek tissue culture chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) or reusable tissue culture growth chambers (11). The test inoculum and cold cell suspension were added to the growth chambers at a ratio of 4 to 1. The test inoculum was diluted 1:5 or 1:10 with Eagle growth medium before being added to individual wells. One 1:5 well and one 1:10 well were inoculated per suspension. The test slides were incubated for 1 h in a humid chamber (34°C) incubator with a 5% CO_2 atmosphere. The medium containing DEAE-dextran was removed and replaced with fresh Eagle growth medium. The slides were incubated under the same conditions for 72 h. At this time the medium and chambers were removed from the slides. The slides were washed once by immersion in phosphate-buffered saline (pH 7.6) for 5 min and air dried. The FAT was then performed. Results were determined as negative (no antigen detected) or positive (the presence of one or more positive cells). The percentage of positive cells was estimated.

RESULTS

Viral isolation and FAT results are shown in Table 1. Nineteen mouse brains were determined to be FAT positive by both microscopists. One mouse brain was determined to be positive by one microscopist and negative by the other microscopist. The MIT produced 18 positive and 2 negative results, and the RTCIT on neuroblastoma cells produced 19 positive results and 1 negative result. The BHK-21 cell RTCIT produced 3 positive and 17 negative results. Sample 11, which produced equivocal FAT, negative MIT, and negative BHK-21 cell RTCIT results, was found to be positive by RTCIT on neuroblastoma cells. Two samples were MIT negative and neuroblastoma cell RTCIT positive, whereas one sample was neuroblastoma cell RTCIT negative and MIT positive. This sample, number 4, killed one of the five inoculated mice. The three samples (8, 12, and 16) that were found to be positive by both RTCIT procedures produced approximately 10 times more infected neuroblastoma cells than BHK-21 cells, After being tested, all samples were frozen at −80°C. When thawed and retested, sample 4 would neither kill mice nor infect neuroblastoma cells.

The presence of 50 μg of DEAE-dextran per ml in the cell suspension inhibited acceptable growth of BHK-21 cells. This result was not seen with neuroblastoma cells. For uniformity, the medium containing DEAE-dextran was replaced with fresh Eagle growth medium for both cell lines after a viral adsorption period of 1 h. With this technique a confluent cell sheet could be obtained in 48 to 72 h with both cell lines.

DISCUSSION

The data presented here indicate that neuroblastoma C-1300 cells are more sensitive for the isolation of fox street rabies virus than are BHK-21 cells. This result may be explained in part by the fact that mouse neuroblastoma cells contain a catalog of surface receptors similar to that in normal mouse brain (13). Being a neurotropic virus, rabies may be expected to attach to plasma membranes of nervous tissue origin more readily than to membranes of nonneural origin. The superior sensitivity of neuroblastoma cells over other cell lines has previously been demonstrated with laboratory-modified strains of rabies and rabieslike viruses (1, 4, 15, 16). Using both primary and continuous cell lines of brain and kidney origin, Umoh and Blenden (16) demonstrated that street rabies virus was more invasive for nervous tissue cell lines than for other cell lines.

The evaluation reported here was undertaken to select a cell line for an isolation procedure that could be used routinely to augment or replace the MIT in rabies diagnosis. To be satisfied that the in vitro procedure was as sensitive as the in vivo procedure, we prepared brain suspensions with a paucity of antigen, placing these suspensions at the margin of sensitivity for the MIT. If an in vitro procedure could detect this amount of virus, we considered it to be a suitable MIT replacement. This small amount of antigen is only occasionally seen in routine rabies diagnosis. More frequently, positive specimens contain large amounts of antigen that are easily detected by the FAT, MIT, and neuroblastoma cell RTCIT. However, weakly positive brain smears represent the condition most commonly misdiagnosed in diagnostic laboratories, as reported previously (3). In that study, 15.6% of the 136 laboratories participating failed to properly diagnose, by using the FAT, weakly positive brain smears. In the present study we reproduced weakly positive brain smears and showed that when properly performed, the FAT, MIT, and neuroblastoma cell RTCIT are equally sensitive in detecting the small amounts of antigen present.

We have found (unpublished data) that the use of parallel titrations is an unsuitable technique for evaluating the sensitivity of an in vitro MIT replacement. We reported previously (12) that BHK-21 cells were as sensitive as the MIT

when performing endpoint dilutions. From those data we concluded that a BHK-21 cell RTCIT would be suitable as an MIT replacement. The results of this present study do not substantiate this conclusion. At higher concentrations of brain tissue, not seen in endpoint dilutions, BHK-21 cells were not as sensitive to rabies virus as was the MIT. The inhibitory effect of 10% brain suspensions on cultured cell growth has previously been reported for BHK-21 cells (12). Smith et al. (14) reported that in their neuroblastoma cell isolation procedure one brain suspension was positive at 1% and negative at 10%, possibly because of the dilution of some inhibitor in the suspension. Koprowski (7) noted that a similar problem occasionally occurred with the MIT when it became necessary to dilute the suspension to demonstrate virus. In our experience a 5- to 10-fold dilution was necessary to remove or suitably reduce the problem.

Our findings support the use of murine neuroblastoma C-1300 cells (clone NA) as a sensitive cell line for the routine isolation of street rabies virus. Further work is warranted to confirm the sensitivity of this cell line to other strains of street rabies virus as well as to demonstrate the practicality of in vitro isolation on a routine basis. This work is presently under way.

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