

# Development and Evaluation of an In Vitro Virus Isolation Procedure as a Replacement for the Mouse Inoculation Test in Rabies Diagnosis

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**A tissue culture virus isolation procedure for rabies street strain virus on mouse neuroblastoma cells is described. Parameters for the optimum sensitivity of the procedure were determined to include a minimum 4-day incubation of virus in tissue culture and the use of diethylaminoethyl-dextran for increased cell susceptibility. The in vitro procedure performed well in a comparison with the fluorescent-antibody test and the mouse inoculation test (MIT) on weakly positive brain tissue. Decomposed specimens and virus inhibitors present in brain suspensions were found to interfere with the in vitro procedure. A Formalin-methanol fixative was found to be superior on plastic 96-well plates to previously used fixatives. A 2-year clinical trial of the procedure in parallel with the MIT demonstrated the practicality of the procedure. Accordingly, the New York State rabies diagnostic laboratory has replaced the MIT with the in vitro procedure as a backup for the fluorescent-antibody test in the routine diagnosis of rabies.**

Exposure to the rabies virus can result in a fatal infection in humans unless the disease is prevented by prompt post-exposure vaccination. The decision to provide or withhold that vaccination is frequently based solely on the results of rabies antigen detection assays performed on brain tissue of the biting animal. The direct fluorescent-antibody test (FAT) has proven to be a fast and reliable diagnostic tool for the routine diagnosis of rabies (6). Success with the FAT has prompted many laboratories to abandon the confirmatory mouse inoculation test (MIT) (9). Experience and proficiency tests (9, 27, 39) have demonstrated that the FAT is not infallible. Therefore, confirmation of immunofluorescence microscopy results by intracerebral inoculation of laboratory mice (13) is the recommended practice. The value of the MIT is its ability to detect small quantities of rabies virus in very weakly positive specimens that can produce false-negative FAT results. A tissue culture procedure that would replace the MIT must be as sensitive.

The BHK-21 and CER cell lines were originally identified as suitable host systems (14, 23, 29). More recently, a murine neuroblastoma cell line (C-1300) was found to have superior sensitivity (10, 22, 28, 34-36).

In this report we describe the determination of optimal conditions for the rabies tissue culture infection test (RTCIT) and a comparison of the RTCIT, FAT, and MIT. We investigated the following parameters associated with the RTCIT procedure: centrifugation of brain suspensions, the effect of brain suspensions on rabies virus infection, optimal incubation period, the use of suspended cells or monolayers, diethylaminoethyl-dextran (DEAE-D; Pharmacia Fine Chemicals AB, Uppsala, Sweden) as an aid to cell susceptibility determination, decomposition of specimens, and the use of fixatives on plastic 96-well plates. In addition to these empirical studies, we evaluated the practicality of the RTCIT by performing the test in parallel with the MIT and FAT on all human exposure specimens submitted to the Rabies Diagnostic Laboratory of the New York State Department of Health from June 1984 through January 1986.

## MATERIALS AND METHODS

**Cell culture and media.** Mouse neuroblastoma cells (C-1300) (1), clone NA (17) (provided by T. J. Wiktor, Wistar Institute, Philadelphia, Pa.), were used at passages 30 to 50. Cells were grown in Eagle minimum essential medium supplemented with 10% fetal bovine serum-10% tryptose phosphate broth-2 mM glutamine-2.2 mg of NaHCO<sub>3</sub> per ml-200 IU of penicillin-0.4 mg of streptomycin per ml (EGM). EGM was used for all tissue and cell suspensions and as a diluent in viral titrations. When the cells achieved 75 to 95% confluency in tissue culture flasks, they were dissociated with trypsin (3 mg/ml) in Hanks balanced salt solution without Ca<sup>2+</sup>, Mg<sup>2+</sup>, or NaHCO<sub>3</sub>. The trypsinized cells were suspended in EGM at room temperature and cooled rapidly in an ice water bath. Repeated trituration during cooling prevented the formation of cell aggregates. The cell concentration was adjusted to 3.25 × 10<sup>5</sup> to 3.5 × 10<sup>5</sup>/ml. Double trypsinization was done by a modification of a previously reported technique (7). Cell suspensions from the first trypsinization procedure were centrifuged at 100 × g for 10 min. The pellet was suspended in 2 ml of trypsin diluent containing 3 mg of trypsin per ml and was incubated at 35°C for 5 min. The reaction was stopped by the addition of 2 ml of fetal bovine serum. The cells were centrifuged and suspended in fresh EGM, and the cell count was adjusted to 3.25 × 10<sup>5</sup>/ml.

To compare virus infection in cell monolayers with that in suspended cells, monolayers were established by seeding wells in 96-well plates 24 h earlier with 1.5 × 10<sup>4</sup> neuroblastoma cells. At the time of challenge the EGM was aspirated and replaced with 0.2 ml of virus suspension. For suspended cell infection, see the discussion of the RTCIT below.

**Virus.** Isolates of rabies virus were obtained from the red fox (*Vulpes fulva*), big brown bat (*Eptesicus fuscus*), striped skunk (*Mephitis mephitis*), and cow. These samples were from animals with naturally occurring infections that were submitted to our diagnostic laboratory. Isolates of rabies virus from raccoon (*Procyon lotor*), Mexican dog, and Arctic fox (*Alopex lagopus*) were supplied by G. Baer (Centers for Disease Control, Atlanta, Ga.). All rabies virus isolates were

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identified and typed by using a panel of monoclonal antibodies produced at the Wistar Institute (41), the Centers for Disease Control (30), and our laboratory. The rabies virus strains from the red fox, bat, cow, and skunk were 10% original brain suspensions. The rabies virus strains from the raccoon, Arctic fox, and Mexican dog were passaged once in mice before 10% brain suspensions were prepared. Brain tissue was thoroughly ground with a mortar and pestle, diluted with EGM, and vortexed in a screw-cap tube.

**FAT.** The FAT was performed by the technique of Dean and Abelseth (6), with the use of modifications described previously (22).

Processing of 96-well plates for the FAT was performed as follows. Growth medium was aspirated from test wells and replaced with phosphate-buffered saline (PBS). After 2 min the PBS was replaced by fixative (see below). Fixation was done for 1 h at room temperature. The FAT was then performed. Fluorescence was examined on an inverted microscope (model IM 35; Zeiss) equipped with a lamp (HBO 100/W2; Osram) and a filter combination (BP 450-490/FT 510/LP 520 KGL; Zeiss). Magnifications for tissue culture were  $\times 100$  and  $\times 300$ , and magnification for mouse brain slip smears was  $\times 320$ .

**MIT.** Virus was quantitated by intracerebral inoculation of virus into weanling mice (13) as described previously (22). Mice were euthanized at the appearance of signs of rabies, and infection was confirmed by the FAT on brain tissue. Mouse intracerebral lethal doses (MICLD<sub>50</sub>s) were calculated by the method of Reed and Muench (20).

**RTCIT.** The in vitro isolation of virus in cell culture was performed in 96-well plates (Corning Cell Wells; Corning Glass Works, Corning, N.Y.). Centrifuged 10% brain suspensions were diluted 1:5 with cold EGM. Suspensions containing less than 10% brain tissue, a result of serial dilution, were added directly to individual wells. Each well received 0.2 ml of test suspension and 0.05 ml of a DEAE-D-sensitized cell suspension. Five replicate wells were prepared for each suspension and dilution. The cells in plates were incubated in a 34°C moist chamber incubator with a 5% CO<sub>2</sub>-95% air atmosphere. After 4 days of growth, the cells were examined by the FAT. Results were determined as negative (no antigen detected) or positive (the presence of one or more cells with intracytoplasmic antigen). When applicable, the percentage of infected cells was an estimate taken from five replicate wells. The 50% tissue-culture infective doses (TCID<sub>50</sub>s) were calculated by the method of Reed and Muench (20).

**Centrifugation.** To assess the effect of brain suspension clarification on the isolation techniques, the following test was performed. Individual 10% brain suspensions were prepared from the three virus isolates and centrifuged at various speeds for 30 min in a refrigerated centrifuge. The supernatant was then assayed by the MIT and RTCIT.

**Fixatives.** The fixative of choice in immunofluorescence microscopy for rabies virus detection is absolute acetone. However, absolute acetone cannot be used as a fixative for cells that are attached to plastic 96-well plates. Therefore, to provide optimal fixation of cells for the FAT on 96-well plates, the following fixatives were evaluated: acetone (50, 80, and 90%) in distilled H<sub>2</sub>O; 80% acetone and 20% Formalin; 10% Formalin in distilled H<sub>2</sub>O; 100% methanol; 50% methanol in distilled H<sub>2</sub>O; 50% methanol and 50% Formalin; and 3% paraformaldehyde in distilled H<sub>2</sub>O. Following the aspiration of EGM, the cell sheets were given a 2-min wash with PBS. The PBS was removed, and the wells were filled with fixative and replaced with fresh fixative after 1 min.

Fixation was done for 1 h at room temperature. The wells containing acetone-H<sub>2</sub>O were emptied and allowed to air dry. Other fixatives were aspirated, and the cells were washed three times in PBS (5 min per wash) and allowed to air dry. The wells that were fixed with 3% paraformaldehyde in distilled H<sub>2</sub>O were subsequently treated with 0.05% Nonidet P-40 (BDH Chemicals Ltd., Poole, England) for 30 min, followed by five 2-min washes with PBS and air drying (15). All wells were then processed by the FAT. Each well was evaluated for immunofluorescence intensity and cellular-antigen morphology.

**Effect of tissue decomposition on test sensitivity.** To determine the effect of decomposition on the sensitivities of the FAT, MIT, and RTCIT, a modification of a previously published procedure (16) was performed. Mice were inoculated intracerebrally with a strain of rabies virus from a skunk. At the first signs of clinical illness, the mice were asphyxiated with CO<sub>2</sub> and stored at -20°C. On day 0 of the procedure, 35 mice were thawed and stored in a biological safety cabinet at 25 ± 2°C. The mice were stored in an open container to minimize an increase in temperature because of decomposition. At 24-h intervals, the brains from five arbitrarily selected mice were removed, pooled, and homogenized. The homogenate was used to prepare slides for the FAT and to prepare suspensions for the MIT and RTCIT. The FAT was scored on a negative and a +1-to-+4 scale. Isolation results were recorded as the number of mice positive/number of mice inoculated and the number of tissue culture wells positive/number of tissue culture wells inoculated.

## RESULTS

**Centrifugation.** The infectious dose titer in both the MIT and the RTCIT depended on the centrifugal force that was used to prepare the supernatant (Fig. 1).

Unspun suspensions adversely affected the growth of neuroblastoma cells in the RTCIT. Tissue fragments appeared to block the attachment of neuroblastoma cells to the growth surface. A 30-min centrifugation at 200 × *g* cleared the suspension sufficiently for satisfactory attachment of the cells and offered the smallest loss of virus among the centrifugal forces tested. All three virus isolates had higher titers in the RTCIT using the samples centrifuged at 200 × *g* than in the MIT using unspun samples.

**Comparing the sensitivities of MIT and RTCIT.** The results of titrations (Table 1) demonstrated that the RTCIT was more sensitive to rabies virus than the MIT was. These results may be misleading, however, since other factors in the original brain suspension were diluted concomitantly. These factors could interfere with or stimulate viral growth in a tissue culture system. The rabies inhibitory substance present in the brains of rabies virus-infected animals, for example, has been identified as immunoglobulin G (8, 18). To present a more valid assay of the sensitivities of the FAT, MIT, and RTCIT procedures, we examined the brains of mice that were sacrificed after rabies virus infection but before the onset of clinical signs of infection (Table 2). This produced a panel of tissues and suspensions that mimicked very weak routine specimens and that taxed the sensitivities of the three procedures.

It was difficult to identify by the FAT the antigen in the brains of mice that were euthanized after 102 h. Classical ringlike inclusions were not seen, and a positive diagnosis was accomplished by determination of the presence of minute antigen particles. The panel of slides and suspensions

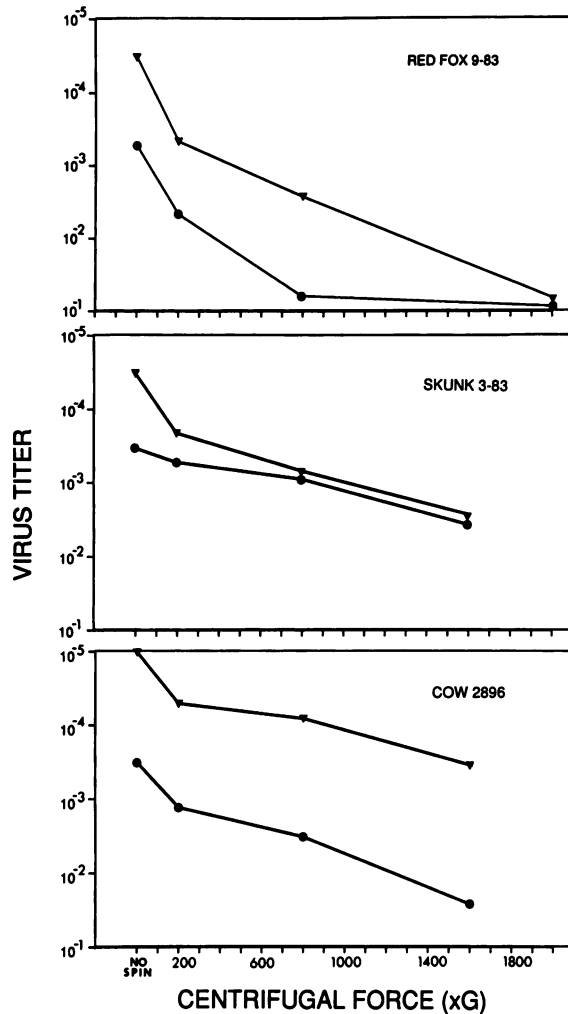


FIG. 1. Effect of a 30-min centrifugation of 10% brain suspensions on virus isolation by the MIT (●) and RTCIT (▲).

at 102 h produced the greatest discrepancy between assays, demonstrating the continued need for FAT backup procedures. The homogenate prepared from brains of mice that were sacrificed 120 h postinoculation presented a more consistent amount of antigen in the FAT. Typically, there were 1 to 5 antigen particles per microscopic field, and small ringlike inclusions were seen occasionally. At 126 h postinoculation, antigen particles ranging in size from dust to classical Negri bodies were seen in every microscopic field.

Results by the MIT and RTCIT procedures were in agreement for 49 of the 59 positive samples. Seven samples were RTCIT positive and MIT negative, and three samples were MIT positive and RTCIT negative. No significant difference in sensitivity ( $P = 0.05$ ) between the two backup procedures could be found ( $\chi^2$ ).

Of the 90 inoculated mice, 59 were positive for rabies virus. This was determined for all mice by at least two procedures. By using the 59 weakly positive samples as a basis for comparison, the sensitivity of the FAT was 93%, that of the MIT was 88%, and that of the RTCIT was 95%. Early diagnosis studies were performed in which three other strains of rabies street strain virus were used (Fig. 2). Although the number of mice tested was small, there was no

significant difference in sensitivities among the three procedures.

**Fixatives.** Specific fluorescence was absent or weak when 50% acetone in  $H_2O$ , 80% acetone and 20% Formalin, or 100% methanol and 50% methanol in  $H_2O$  were used as the fixatives. Plastic 96-well plates were crazed by 90% acetone in distilled  $H_2O$ . An 80% solution of acetone in distilled  $H_2O$  produced satisfactory results; however, occasional opaqueness in the clear plastic was experienced. The procedure with paraformaldehyde as the fixative produced results comparable to those obtained with 80% acetone in distilled  $H_2O$ , without damaging the plastic. Formalin proved to be a satisfactory fixative for the tissue culture procedure on plastic; however, the absolute methanol-Formalin (1:1) fixative was superior to all other fixatives tested. With the absolute methanol-Formalin fixative, the cellular morphology was equal to that seen with Formalin-fixed cells, and there was a greater resolution of antigen morphology.

**Comparison of monolayers with suspended cells.** To compare the susceptibilities of trypsinized cells, double-trypsinized cells, and established cell sheets to rabies virus infection, parallel titrations of virus were performed in 96-well plates. Cell monolayers have been shown to be more sensitive to rhabdovirus infections than freshly trypsinized cells are (21). Single or multiple trypsinizations remove various glycopeptide fractions from neuroblastoma membranes (7). Carbohydrate-containing moieties have been identified as putative receptors for rabies virus (31, 34, 42).

Titration results indicate that neuroblastoma susceptibility to rabies street strain virus is unaffected by the choice of monolayers or single- or double-trypsinized cells in the RTCIT. Endpoint titers of three strains of virus were affected by no more than 0.05  $\log_{10}$  TCID<sub>50</sub> per 0.2 ml. These results agree with those of an earlier study in which CVS virus was used (35).

**Viral inhibitory substances in brain suspensions.** Incubation of rhabdoviruses with gangliosides and phospholipids, which are present in brain suspensions, reduces the percentage of infected cells during in vitro assays (5, 24, 31, 32).

Two percent suspensions of normal rabbit and mouse brain were clarified by centrifugation at 1,000  $\times g$  for 1 h, filtration, or a combination of the two methods. By using EGM and the suspensions described above as diluents, rabies street strain virus was titrated by the RTCIT and MIT.

The brain tissue suspensions that were used as the diluent for the RTCIT interfered with cell infection. A 2% rabbit brain suspension that was clarified by centrifugation at 1,000  $\times g$ , when used as a titration diluent, reduced the endpoint titer by a factor of  $10^2$  compared with that when EGM was used as the diluent (Table 3). An average titer loss of  $10^2$  (range,  $10^{1.54}$  to  $10^{2.52}$ ) was produced when a 2.0% mouse brain suspension was used as the diluent after the suspension was clarified by centrifugation at 1,000  $\times g$  and filtration of the supernatant on a 0.45- $\mu m$ -pore-size grid (Table 4).

TABLE 1. Parallel titration results for five strains of rabies virus by the MIT and RTCIT isolation procedures

Virus strain (source)	$\log_{10}$ MICLD <sub>50</sub> per 0.03 ml	$\log_{10}$ TCID <sub>50</sub> per 0.20 ml
6-83 (red fox)	4.24	4.83
38-82 ( <i>Eptesicus</i> bat)	2.63	4.52
39-82 ( <i>Myotis</i> bat)	1.67	4.83
S.E. (raccoon)	4.16	6.20
Mexican dog	5.42	6.60

TABLE 2. Results of early diagnosis test comparing the FAT, MIT, and RTCIT for rabies virus performed on brains of mice injected with a virus of red fox origin<sup>a</sup>

Time (h) postinoculation	No. positive <sup>b</sup>	No. of mice with the following test results:			
		FAT <sup>+</sup> , MIT <sup>+</sup> , RTCIT <sup>+</sup>	FAT <sup>+</sup> , MIT <sup>+</sup> , RTCIT <sup>-</sup>	FAT <sup>+</sup> , MIT <sup>-</sup> , RTCIT <sup>+</sup>	FAT <sup>-</sup> , MIT <sup>+</sup> , RTCIT <sup>+</sup>
102	14	6	0	4	4
120	22	18	2	2	0
126	23	22	0	1	0

<sup>a</sup> Rabies virus-infected mice were euthanized before the onset of clinical signs and were used as a source of weakly positive diagnostic samples. A total of 90 mice were inoculated intracerebrally with 50 to 100 MICLD<sub>50</sub>s of virus per 0.03 ml; and 30 mice were harvested at 102, 120, and 126 h postinoculation.

<sup>b</sup> Number of mice of 30 inoculated mice that were positive at the indicated times. All mice were positive by at least two of the procedures.

<sup>c</sup> FAT results are the consensus of the evaluation of the same microscope slides by three microscopists.

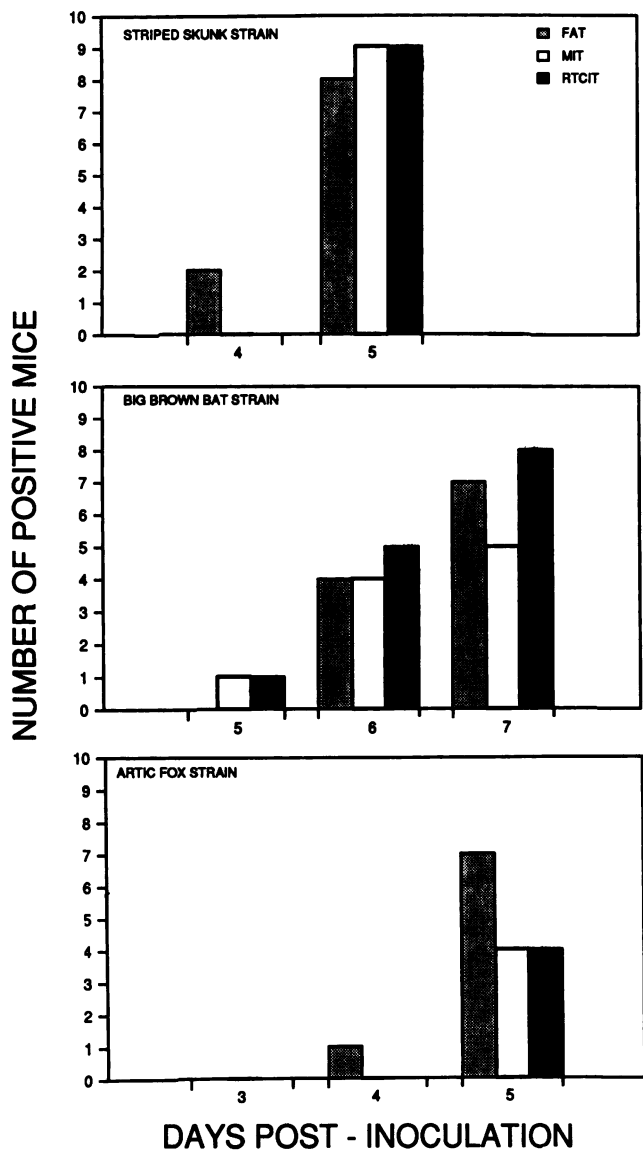


FIG. 2. Early diagnosis test results. Mice (20 mice were inoculated with skunk virus, and 30 mice were inoculated with virus from a bat or fox) were inoculated intracerebrally with 50 to 100 MICLD<sub>50</sub>s of virus per 0.03 ml. On the days indicated, 10 mice were euthanized and their brains were tested by the FAT, MIT, and RTCIT.

**DEAE-D.** DEAE-D has been shown to aid virus invasiveness in cell culture (2, 12, 14, 23). The effect of DEAE-D on the susceptibility of neuroblastoma cells to rabies virus infection was assayed by parallel titration. Neuroblastoma cells were added to plates at a concentration of  $1.5 \times 10^3$  per well. A second set of plates received cells that were pre-treated with 25 µg of DEAE-D per ml.

Comparison of endpoints suggested that the polycation does not significantly alter the susceptibility of neuroblastoma cells to the virus strains that were used (Table 4). However, when the percentage of infected cells tested with and without the polycation was measured, DEAE-D was shown to produce up to a 10-fold increase in the number of infected cells at comparable viral dilutions. Unlike results seen with BHK-21 cells (12, 23), the polycation DEAE-D was not toxic to neuroblastoma cells at the concentration used and, thus, could be left on the cells for the duration of the experiment.

**Optimal incubation period.** The MIT may take 15 to 30 days or longer (33, 40) to demonstrate virus in a weakly positive specimen compared with the 1 (3, 4, 19) to 5 (22, 23, 38) days needed for the RTCIT. Thus, an advantage of the RTCIT is the shorter period required to obtain final results. An RTCIT was performed on five replicate titrations of rabies virus of red fox origin using clarified 2.0% normal rabbit brain as the diluent. On days 1 to 5, a titration was examined by the FAT. The results of this test are presented in Fig. 3. The original 10% suspension of rabies virus-infected brain and the  $10^{-1}$  dilution were readily diagnosed as positive on days 1 to 5, with 95 to 100% of the inoculated wells containing virus. At greater dilutions of virus ( $10^{-2}$  and  $10^{-3}$ ), the sensitivity of the RTCIT dropped to unacceptable levels when incubation was done for 1 or 2 days. In the dilutions containing the smaller amounts of virus ( $10^{-2}$  and  $10^{-3}$ ), the greatest number of infected wells was seen on day

TABLE 3. Effect of inhibitory substance in normal brain suspensions determined by virus titration with the diluents EGM or brain suspensions<sup>a</sup>

Procedure	Log <sub>10</sub> TCID <sub>50</sub> with the following diluents:	
	EGM	2% rabbit brain <sup>b</sup>
RTCIT	4.66 <sup>c</sup>	2.68 <sup>d</sup>
MIT	3	2.7

<sup>a</sup> Virus was of red fox origin.

<sup>b</sup> The 2% rabbit brain diluent was clarified by centrifugation at  $1,000 \times g$  for 30 min.

<sup>c</sup> Value is the average of five separate experiments (range,  $10^{4.83}$  to  $10^{4.50}$  TCID<sub>50</sub> per 0.03 ml).

<sup>d</sup> Value is the average of four separate experiments (range,  $10^{2.83}$  to  $10^{2.49}$  TCID<sub>50</sub> per 0.03 ml).

TABLE 4. Effect of DEAE-D and brain suspensions on the ability of neuroblastoma cells to isolate rabies street strain virus

Virus source	Log <sub>10</sub> TCID <sub>50</sub> per 0.2 ml <sup>a</sup>	
	With DEAE-D	Without DEAE-D
Skunk	0.91/2.62	0.83/2.71
Red fox	2.29/3.83	2.14/4.38
Raccoon	1.16/3.38	0.9/3.42

<sup>a</sup> Parallel titrations in 96-well plates were assayed by the RTCIT. The first number is the result when the diluent used for titration was a 2% normal mouse brain suspension clarified by centrifugation at 1,000 × g for 1 h followed by filtration of the supernatant on a 0.45-μm-pore-size grid. The second number is the result when the diluent for titration was EGM.

4. The titer of the undiluted viral suspension rose from 10<sup>1.5</sup> on day 1 to 10<sup>2.40</sup> on day 4.

**Effect of decomposition on test sensitivity.** The results of the FAT, MIT, and RTCIT on progressively decomposed rabies virus-positive tissue are given in Table 5. The FAT was a more sensitive procedure than either of the isolation procedures. After 4 days of decomposition, brain tissue was toxic to neuroblastoma cells and infected only one of five inoculated mice. On day 5 both isolation tests were unsatisfactory. On days 4 and 5 the FAT recorded a positive (2+) result; however, the FAT microscope slides may have been unsatisfactory for a reliable negative determination because of the excessive loss of tissue during the PBS washing step.

**Clinical trials.** The RTCIT was performed in addition to our routine diagnostic FAT and MIT on human exposure specimens during the years 1984 and 1985. Ten percent brain suspensions were centrifuged at 200 × g for 15 min. The positive control consisted of five wells inoculated with a rabies street strain virus suspension containing approximately 5 to 10 TCID<sub>50</sub> per 0.2 ml and five wells each inoculated with 1:10 and 1:100 dilutions of this suspension. During the years 1984 and 1985 specimens from a total of 2,112 animals that were negative for rabies virus by the FAT were examined in parallel by the RTCIT and MIT. The specimens were obtained from 918 cats, 839 dogs, 154 raccoons, 98 bats, 44 bovines, 25 ferrets, 15 foxes, 14

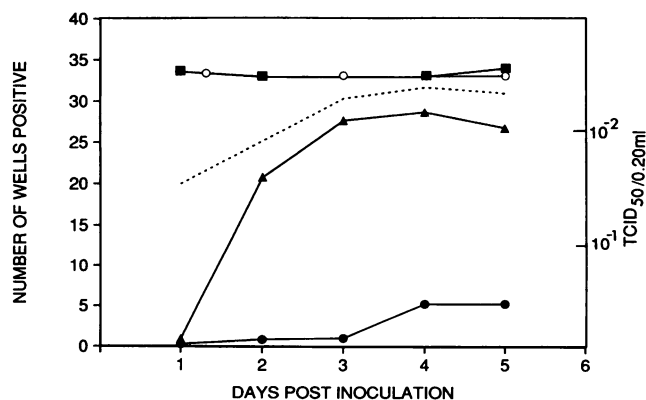


FIG. 3. Results of isolation of rabies virus from red fox by the RTCIT in 96-well plates. Five replicate titrations (34 wells per dilution) were prepared by using a clarified 2.0% normal rabbit brain suspension as the diluent. Solid lines represent the number of positive wells per dilution at days 1 to 5. Symbols for dilutions: ■, 10<sup>0</sup>; ○, 10<sup>-1</sup>; ▲, 10<sup>-2</sup>; ●, 10<sup>-3</sup>. The broken line represents the TCID<sub>50</sub> endpoint titer of the undiluted viral suspension at days 1 to 5.

TABLE 5. Diagnosis by the FAT, MIT, and RTCIT of positive tissue that underwent progressive decomposition<sup>a</sup>

Decomposition time (days)	Fat intensity (1+ to 4+)	Notes <sup>b</sup>	MIT results (no. of mice positive/five mice inoculated)	RTCIT results (no. of wells positive/four wells inoculated)
0	3+	1	5	4
1	3+	1	5	4
2	3+	1	5	4
3	2+	2, 3	3	1
4	2+	3, 4	2	Toxic
5	2+	4, 5	0	Toxic
6	2+	4, 5	0	Toxic
7	2+	6	0	Toxic

<sup>a</sup> Euthanized rabies virus-infected mice were held at 25 ± 2°C. At 24-h intervals five mouse brains were pooled, homogenized, and tested for evidence of viral antigen and infectious virus.

<sup>b</sup> Notes: 1, good antigen integrity; 2, antigen started to loose margin integrity; there was at least a 50% drop in fluorescence intensity; 3, brain tissue liquid before homogenization and foul odor; 4, quantity of fluorescing antigen was not diminished, fluorescence intensity was very weak; 5, tissue held very poorly to glass slide in the FAT, and during washes in PBS the majority of tissue was lost; 6, weak fluorescence still detectable by the FAT on tissue fragments that adhered to the microscope slide after washing.

skunks, 3 mink, and 2 horses. All specimens were negative for rabies virus by rabies virus isolation procedures. During the same period, 278 specimens were positive for rabies virus by the FAT. In all cases virus was isolated by the MIT and RTCIT. Of the 2,390 specimens tested by the RTCIT during this period, 72 (3.0%) had unsatisfactory results. Unsatisfactory RTCIT results were most frequently attributable to the decomposition of specimen tissues. Neuroblastoma cell lysis and clumping were the main reasons for unsatisfactory results. No correlation between the method of euthanasia of the animals and unsatisfactory RTCIT results could be found. Since 1 April 1986, our diagnostic laboratory has processed all brain suspensions by the RTCIT, and we have replaced the MIT with the RTCIT as our backup procedure for the FAT.

## DISCUSSION

The results of this study indicate that the RTCIT can be successfully implemented in a routine rabies diagnostic laboratory. The RTCIT has proven to be a reliable procedure that is as sensitive as the MIT to small amounts of virus.

Infection-inhibiting factors in brain suspensions reduce the sensitivity of the RTCIT procedure. While evaluating the RTCIT, we identified the presence of these factors as crucial for performing a reliable comparison of the RTCIT and MIT. Previously, in a procedure involving parallel titrations with EGM as the diluent, an RTCIT with BHK-21 cells was as sensitive to small amounts of rabies virus as the MIT was (23). In subsequent tests with a virus inoculum containing 10% brain tissue, the BHK-21 RTCIT was inferior in sensitivity when compared with that of the MIT on the same inoculum (22). The use of neuroblastoma cells in the RTCIT allowed the procedure to be as sensitive to rabies virus infection as the MIT was. However, the same inhibition by brain tissue was observed with the neuroblastoma cells. A parallel titration performed by the RTCIT with EGM as the diluent produced virus titers that were 1.66 log<sub>10</sub> units higher than those obtained by the MIT (Table 3). When the diluent for the parallel titrations was a 2.0% normal rabbit brain suspension, the RTCIT produced a result equal to that of the

MIT (Table 3). Using three strains of rabies virus and a different source of inhibitor (mouse brain), we again noted this inhibition reaction (Table 4). Note that the MIT showed only a slight ( $0.3\text{-log}_{10}$ -unit) loss of titer because of the effect of a brain suspension diluent, while the RTCIT demonstrated a  $2\text{-log}_{10}$ -unit loss of titer.

If the inhibitory factors could be selectively removed, the RTCIT would potentially be 1 to  $2\text{ log}_{10}$  units more sensitive. Selective removal of these factors with enzymes or detergents could possibly inactivate virus since the rabies virus envelope contains many of the same lipids found in plasma cell membranes (25). However, Shope et al. (26) have demonstrated that herpesvirus can be isolated in tissue cultures that were inoculated with intact cells that were trypsinized from pieces of infected brain tissue. Herpesvirus could not be isolated from a homogenized suspension prepared from the same brain tissue. The protection of intracellular virus by infection-inhibiting substances was postulated as one explanation for these results. This technique should be investigated in future rabies virus isolation studies.

We reported the optimal incubation period for the RTCIT to be 4 to 5 days. Using similar procedures, other investigators (3, 4, 19) have reported that a 24-h incubation is adequate. We noted that a 24-h incubation period is satisfactory only when the test inoculum contains adequate amounts of virus (Fig. 3). In our study more than  $10^{1.5}$  TCID<sub>50</sub> of virus per 0.2 ml was required for positive RTCIT results at 24 h. Lower concentrations of virus could not be detected without a 2- to 4-day incubation. In the early diagnosis test (Table 2), we examined brain tissue which contained various concentrations of virus. Tissue samples which yielded more than  $10^{1.5}$  TCID<sub>50</sub> of virus by the RTCIT were readily diagnosed by the FAT. The quantity of antigen present in these samples was enough to expect a properly functioning FAT, containing controls for specificity (37), to readily detect antigen. An RTCIT that is allowed to incubate for 24 h can therefore only confirm most FAT-positive results. If the purpose of the RTCIT is to detect false-negative FAT results, it is essential that it be as sensitive as possible. At present this mandates a 4-day incubation period.

The performance of the RTCIT in plastic 96-well tissue culture plates provides for an economical alternative to tissue culture growth chambers attached to glass microscope slides when large numbers of suspensions are processed. However, absolute acetone cannot be used as a fixative for cells that are attached to a plastic plate. Formalin has previously been described (19) as a fixative for an RTCIT. Other investigators (11) have found Formalin to be unsatisfactory as a fixative in an *in vitro* immunofluorescence virus isolation procedure. However, the technique did not include a washing step after fixation; we found this process to be essential. In a study of intracellular structures (15), additional treatment of Formalin-fixed membranes has been reported to be necessary for the penetration of antibodies and lectins. In our laboratory and those of other investigators (19) such treatment has been demonstrated to be unnecessary for tissue cultures infected with rabies virus. However, we determined that fixation of the membranes with a methanol-Formalin combination is superior to that with Formalin alone. Methanol, a lipid solvent, possibly acts to permeabilize the membrane and to enhance penetration of the stain.

The 2 to 10% increase in the number of infected cells we found when we used the polycation is in agreement with

other reports of the effect of DEAE-D in determining rhabdovirus infections in other cell systems (2, 12, 23, 42). The actual benefit of this slight increase in susceptibility is a greater ease in locating foci of infection in a weakly infected tissue culture monolayer; what was 1 positive cell without DEAE-D was frequently found to be a 5- to 10-cell plaque when DEAE-D was used. This is important if the RTCIT is to be of value in isolating virus from the most difficult diagnostic specimens.

Our results on the diagnosis of rabies virus in decomposed tissues agree with those of an earlier report on the performance of the FAT and MIT (16). Additionally, we found that the tissue culture procedure is less tolerant to decomposed tissue than either the FAT or MIT is. After 4 days of decomposition, the tissue suspension was toxic to tissue culture growth. Slip smears prepared from the same tissue and processed by the FAT presented a marked loss of fluorescence intensity. At this stage of decomposition, tissues were shown to be unsuitable for reliable rabies virus diagnosis. Positive test results from deteriorated tissues were reliable, as false-positive results were not observed.

A 1979 decomposition study (unpublished data) presented different results. Mice were allowed to decompose in a sealed plastic container. While the mice desiccated with time in the present study, the mice appeared to undergo an anaerobic, moist decomposition in the earlier study. In the earlier study the FAT was negative on day 4, but the MIT and BHK-21 RTCIT still detected virus on day 7. Such variability demonstrates that negative FAT, MIT, or RTCIT results from decomposed tissue could be unreliable and should not be used to justify the withholding of postexposure prophylaxis in humans.

We reported the results of 2 years of clinical trials with the RTCIT in parallel with the FAT and MIT, along with our experience of 30 months of use of the RTCIT as our sole FAT backup procedure. Our diagnostic laboratory has not recorded a false-negative FAT result during this period. For this reason a weak positive control that was titrated to the endpoint in each RTCIT allows for continued monitoring of neuroblastoma cell susceptibility. After several years of such monitoring, we found it necessary to replace cells twice because of the decreased susceptibility of the cell line. On both occasions tests for mycoplasmas were negative and the cell lines were at high passage levels (passages 47 and 50).

For laboratories that are equipped for tissue culture work, the RTCIT offers a fast, sensitive alternative to the MIT. Prospects for future improvements of the RTCIT include steps that would reduce the time necessary for a final result without the loss of sensitivity. We are examining other clones of neuroblastoma cells for variations in their sensitivities to rabies virus. Other investigators (35) have found evidence of variable sensitivities to rabies virus in different clones of the same parent cell line. Mouse neuroblastoma cell line C-1300, clone NA, is a subclone of Neuro-2a (ATCC CCL131) (17). Preliminary comparisons of the Neuro-2a cell line indicate that it is more permissive to viral growth than the NA cell line is. When parallel titrations are performed, the endpoint titers in the two cell lines are identical. However, the percentage of infected cells in comparable dilutions is consistently 5 to 10 times higher in the Neuro-2a cell line.

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