A Tissue Culture Infection Test in Routine Rabies Diagnosis

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ABSTRACT

A cell culture infection test was developed for the isolation of rabies virus from field cases submitted for rabies diagnosis. The procedure involved the addition of a suspension of suspect brain tissue to a suspension of murine neuroblastoma cells in 96-well microtiter plates. The cultures were then incubated at 35-36°C for four days at which time they were fixed, stained with a fluorescein-labelled hamster antirabies antibody conjugate and examined with a fluorescence microscope. Rabies antigen in cells was readily visible as brilliant, apple-green fluorescent particles. This technique was compared with the standard mouse inoculation test and was at least as sensitive to infection with small amounts of virus, required a much shorter test period and was substantially more economical than the mouse inoculation test. The new cell culture test is now in use at this laboratory, replacing the mouse inoculation test.

Key words: Rabies, field virus, isolation, neuroblastoma cells, mouse inoculation.

RÉSUMÉ

Cette étude consistait à développer un test d'infection de cultures cellulaires, dans le but d'isoler le virus de la rage à partir de cas suspects, soumis au laboratoire pour fin de diagnostic. Le procédé impliquait l'addition d'une suspension de tissu cérébral suspect à une suspension de cellules de neuroblastome murin, dans des plateaux de microtitration de 96 puits. Les cultures cellulaires subissent ensuite une incubation de quatre jours, à 35-36°C, la fixation, la coloration avec des anticorps contre le virus de la rage, prépares chez le hamster et conjugués avec de la fluorescéine, ainsi que l'examen au microscope à fluorescence. L'antigène rabique présent dans les cellules se voyait facilement, sous la forme de particules fluorescentes, d'un vert pomme brillant. À la suite d'une comparaison avec le test standard de l'inoculation de souris, cette nouvelle technique se révéla au moins aussi sensible à l'infection, avec une faible concentration de virus; elle permit d'obtenir des résultats beaucoup plus rapidement et elle s'avéra substantiellement plus économique. L'Institut de recherches sur les maladies animales de Nepean l'utilise maintenant, à la place de l'inoculation de souris.

Mots clés: rage, virus des rues, isolement, cellules de neuroblastome, inoculation de souris.

INTRODUCTION

All specimens received at this laboratory for rabies diagnosis are examined by the fluorescent antibody staining technique (FAT) (1) to determine the presence or absence of rabies virus infection. Those specimens which are FAT negative and have had contact with humans are also inoculated into experimental mice. These mice are held for a period of 30 days during which any mice that die are examined by the FAT. The mouse inoculation test (MIT) (2) has been the recommended back-up procedure since the early days of rabies diagnosis.

Because of the observation period (30 days) required for the MIT, the increasing cost of this test, and the need to replace the use of animals in the laboratory, alternative techniques have been sought. Recent developments in cell culture and virus isolation techniques have prompted us to examine these in an attempt to produce a test which can replace the MIT.

MATERIALS AND METHODS

CELL SUSPENSION

A murine neuroblastoma (NA) cell line obtained from Dr. T. Wiktor, The Wistar Institute, Philadelphia, Pennsylvania was grown in MEM-10 medium consisting of Eagle's minimum essential medium (Flow Laboratories) supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth (Gibco), 2 nM l-glutamine (Gibco) and 50 μg/mL neomycin sulphate (Gibco) at 37°C in a moist atmosphere containing 5% CO_2. Prior to use in the virus isolation procedure, the cells were trypsinized with trypsin-EDTA (Gibco) and resuspended in MEM-10 to a concentration of 5×10^5 cells/mL. DEAE-dextran (Sigma Chemical Co.) was added to the cell suspension just prior to the addition of brain suspension to give a final concentration of 25 μg/mL.

BRAIN SUSPENSION

All tissues were received at this laboratory as routine diagnostic specimens and originated from Ontario, Quebec and the Northwest Territories. They represented a variety of terrestrial mammals. A 10% brain suspension was prepared in physiological saline containing 500 IU penicillin G and 2 mg streptomycin sulphate/mL and allowed to settle at 4°C for at least 1 h. A further 1:10 dilution of the upper clear layer was made in MEM-10 and this was used as the inoculum.

RABIES TISSUE CULTURE INFECTION TEST (RTCIT)

For each specimen, 0.1 mL of the prepared NA cell suspension was added to each of four wells in a 96-well microtiter plate (Nunc). To each of the four wells, 0.2 mL of the brain suspension

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Submitted October 31, 1986.

(at 10^{-2}) was added. A total of 24 specimens could be tested with each plate. The cell-tissue suspensions were mixed and the plate incubated at 35-36°C (in 5% CO_2) for four to five days. A single five-well strip of a SAS plate (Lux) was used to prepare controls containing either infected or uninfected cells.

Following incubation, the medium was removed, the wells washed with phosphate-buffered saline (pH 7.2-7.4) and the cells fixed by the addition of 75-80% cold acetone for 30 min at room temperature. The cultures were stained for 30 min at 37°C with a fluorescein-labelled hamster anti-CVS rabies serum conjugate prepared in this laboratory (3). Following rinsing with saline, the cultures were counterstained with 1:200 Evan's Blue for 5-10 min and rinsed in saline. The plates were inverted and the cell cultures examined using a Leitz Orthoplan fluorescence microscope with epi-illumination and a 10 X objective.

COMPARISON OF RTCIT AND MIT

The sensitivity of the RTCIT was compared to the MIT to determine its suitability as a replacement for the latter.

1) The routine diagnosis of rabies in field cases submitted to this laboratory involves the examination of all specimens with the fluorescent antibody staining technique (FAT) and the subsequent intracerebral inoculation of experimental mice with tissues from those FAT negative specimens with which there has been human involvement. Such specimens were ground in a mortar and pestle and resuspended in physiological saline to make a 10% suspension. Five mice were each inoculated intracerebrally with 0.03 mL of this suspension (10^{-1}). For the RTCIT, a 10^{-2} dilution was used.

2) Rabies positive (FAT+) tissue suspensions were inoculated into cell cultures at a 10^{-2} dilution and experimental mice at a 10^{-1} dilution.

3) In an attempt to simulate conditions in which the amount of virus in the suspect tissue is small, 10% suspensions of FAT-positive tissues were prepared and the virus content was determined by titration on NA cells. That dilution which infected only 1-5% of the cells was then prepared and inoculated into mice and NA cells at that same dilution.

RESULTS

During the development of this technique, various procedures were attempted and modified. Although several different cell types have been used in experimental studies on rabies virus, a murine neuroblastoma (NA) and a baby hamster kidney (BHK-21) cell line are the two most commonly used (4-7). Most fixed or laboratory strains of rabies virus replicated readily in both NA and BHK-21 cells, but not all field virus strains replicated equally well in BHK-21 cells. The virus group found in the Northwest Territories, Ontario and Quebec (8) did not replicate easily in BHK-21 cells (unpublished data). On the other hand, NA cells were readily infected with all North American strains of virus examined as well as some from Europe, Africa and South America. The addition of DEAE-dextran to the cell suspension enhanced infection (9).

Infection in NA cells could be demonstrated as early as 24 h when the inoculum contained a high virus content. However, tissues containing small amounts of virus (as would be expected with FAT false-negative specimens) sometimes did not produce demonstrable infection until three to four days postinfection. Following the examination of replicate cultures an incubation period of at least four days was determined to be sufficient to diagnose these infections.

Neuroblastoma cells are susceptible to toxic and other substances associated with brain tissues recovered from field cases. The preparation of an inoculum with a final dilution of 10^{-2} was required to effectively eliminate most, if not all, of these factors and to ensure good cell growth. It was also found that the number of infected cells was as great or greater at an inoculum concentration of 10^{-2} than at 10^{-1}.

During the course of the comparison trials, 3800 FAT negative specimens were inoculated into both experimental mice (at 10^{-1}) and cell cultures (at 10^{-2}). Of these, four were shown to be rabies positive by the RTCIT alone and two by both tests (Table I). One of the latter was very weakly FAT positive when the original tissue was retested. The four specimens that were RTCIT positive and MIT negative were all FAT negative on retest.

One hundred and forty-five FAT positive tissues were inoculated into both cell cultures (10^{-2}) and experimental mice (10^{-1}) (Table I). The inoculum from seven specimens killed both cell cultures and mice early and a diagnosis was not possible. Four specimens were RTCIT positive:MIT negative and two were RTCIT negative:MIT positive. In the former cases, the mice died early of nonspecific causes and in the latter cases, the cells underwent cytolysis. The remaining specimens were positive by both tests.

In the dilution trials to simulate tissues containing small amounts of virus, 159 tissues were tested (Table I). Eleven specimens were negative and 59 were positive by both techniques. Eighty-seven were RTCIT positive and MIT negative. All suspensions had been stored at -20°C between the initial titration and the final test. It has been demonstrated (unpublished data; 10) that a considerable loss of infectivity of rabies virus occurs in brain tissue stored at -20°C.

TABLE I. Comparison of RTCIT and MIT in the Diagnosis of Rabies

Inoculum	No. Tested	Results			
		RTCIT-/MIT-	RTCIT+/MIT-	RTCIT-/MIT+	RTCIT+/MIT+
FAT negative	3800	3794	4	0	2
FAT positive	145	7[a]	4[b]	2[c]	132
FAT positive dilutions	159	11	87	2	59

[a]Both cells and mice killed early due to contamination

[b]Mice died early due to contamination

[c]Cells underwent cytolysis due to contamination

DISCUSSION

While BHK-21 cell cultures have been and continue to be useful for many studies, it has become evident that not all rabies virus strains replicate equally well in these cells. On the other hand, murine neuroblastoma cells have been shown to be an effective cell line for the isolation and growth of those rabies virus groups so far studied at this laboratory. Infection with even small amounts of virus produces immunofluorescence in aggregates of cells when stained by the fluorescent antibody staining technique. A definitive result, either positive or negative can be obtained in four days. Most specimens that are negative by the FAT and positive by the MIT have very small amounts of viable antigen present and the incubation time in mice is extended to periods of 12-20 days (11). Those specimens which remain rabies negative by the MIT cannot be reported until the completion of the 30-day test period.

The mouse inoculation test has been assumed to be at least as sensitive as the fluorescent antibody staining test for the demonstration of rabies virus (10,12,13,14) depending upon such factors as the amount of virus present, the condition of the suspect tissue, the quality of reagents and the expertise of the laboratory personnel. Most previous comparisons have been made with specimens which were FAT negative and subsequently inoculated into mice. At this laboratory, approximately 35,300 such specimens have been inoculated into mice during the past eight years; only 29 (0.8%) of these were proven rabies positive by the MIT. In the present study, only two of 3800 (0.05%) were proven rabies positive by the MIT. However, in the dilution trial in which the inoculum for both tests was at the same dilution, approximately 50% of known rabies positive specimens failed to produce mortality in mice although viable virus was demonstrated with cell cultures (Table I). This indicates that the RTCIT is considerably more sensitive to infection with low amounts of virus than are experimental mice. Low viral concentrations would be expected in those specimens which are falsely negative by the FAT. This was further substantiated by the finding of four RTCIT positive specimens (from the 3800 FAT negative specimens) which were negative by both the FAT and MIT.

The use of a 10^{-2} dilution of the original brain tissue was optimum for isolation of field virus in NA cells. Problems associated with cytolysis due to bacterial and/or toxic contaminants were effectively eliminated except in a few cases where brain autolysis was severe. The reason(s) for similar or increased numbers of infected cells at 10^{-2} as compared to a 10^{-1} dilution cannot be presently explained.

The fact that viable virus was not found in 11 of the 159 dilution trial specimens illustrates the loss of infectivity which can occur at temperatures of -20°C or higher. The dilution of the virus in these particular suspensions was to such an extent that freezing following the initial titration rendered the virus noninfective. These findings substantiate those of Wachendorfer *et al* (10) that the long-term storage of virus samples should be at considerably lower temperatures.

This laboratory currently tests some 10,000-13,000 specimens each year of which approximately 5,000-6,000 involve contact with humans and are rabies negative by the fluorescent antibody staining test necessitating further testing. Our objective was to develop a technique which could replace the standard mouse inoculation test and be a practical test for use in a laboratory handling large numbers of specimens. It has been shown that the RTCIT is at least as sensitive as the MIT for the isolation of rabies virus. Results are obtained in four to five days in the former compared with 15-30 days in the latter test. The actual time required to perform the two tests is approximately equal. The RTCIT is substantially more economical in both human and fiscal resources. Finally, an annual reduction in the use of approximately 30,000 mice in this laboratory is anticipated. This test was officially adopted as a replacement for the MIT in this laboratory on June 1, 1986.

ACKNOWLEDGMENTS

The capable assistance of Mr. Steve Miller is gratefully acknowledged as is that of Miss K. MacKenzie, Mr. D. Rasmussen and Mr. B. Ullett. The author is grateful to Mr. R. Rudd, New York State Department of Public Health, Albany, New York and Dr. P. Rollin, Pasteur Institute, Paris, France for a continued exchange of information.

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