Rabies Antibody Determination by Immunofluorescence in Tissue Culture[1]

JOHN G. DEBBIE, JEROME A. ANDRULONIS, AND MELVIN K. ABELSETH

*Division of Laboratories and Research, New York State Department of Health,
Albany, New York 12201*

A tissue culture (TC)-fluorescent antibody (FA) technique for the measurement of rabies-neutralizing antibody was found to be reliable and comparable to the standard mouse serum neutralization test. This test was performed with BHK-21 cells infected with the ERA vaccine virus strain on Lab-Tek TC chamber slides. A Flury high egg passage (HEP) rabies virus strain grown on continuous line of African green monkey (Vero) and on BHK-21 cells was investigated to determine its utilization in a TC-FA neutralization procedure. Although both the HEP and ERA viruses infected Vero and BHK-21 cells, the amount of fluorescent antigen observed was most consistent with ERA virus and BHK-21 cells.

Although primary cultures and established cell lines of non-nervous tissue have been used for rabies virus propagation with varying degrees of success (2, 4–7, 9, 10, 12, 13, 16, 18), intracerebral inoculation of young adult mice with serum-virus mixtures has remained the accepted method of determining serum-neutralizing antibody titers (17).

This paper describes a tissue culture method for measuring rabies serum antibody utilizing BHK-21 cells, the ERA strain of rabies virus, and the fluorescent antibody test (FAT).

MATERIALS AND METHODS

Tissue culture. The BHK-21 cell line was maintained at the Division of Laboratories and Research, N.Y. State Department of Health. The cells were grown on tissue culture (TC) chamber slides (Lab-Tek Products, Division Miles Laboratories, Inc., Westmont, Ill.) in an atmosphere of 5% CO_2. Each chamber was seeded with 0.4 ml of a TC suspension containing approximately 75,000 cells/ml. Eagle medium was used with the addition of 10% fetal bovine serum (FBS) and 10% tryptose phosphate broth for culture growth. The FBS was reduced to 3% for culture maintenance.

Virus. The commercial, lyophilized ERA vaccine strain of rabies virus was received from Jensen-Salsbery Laboratories, Kansas City, Mo., and was reconstituted to 2 ml with TC maintenance medium. This virus had a mouse intracerebral median lethal dose (MICLD$_{50}$) titer of $10^{3.5}/0.03$ ml in 10 to 12 g of NYLAR mice (N.Y. State Department of Health, Division of Laboratories and Research, strain).

FAT. After the chambers were detached, the slides were acetone-fixed for 1 hr at 4 C. The FAT was performed essentially as previously described (3, 8).

Serum neutralization tests. Sera from foxes and hamsters obtained for other experimental studies were utilized in serum neutralization (SN) tests. Additional animal and all human sera were supplied by the Center for Disease Control, Atlanta, Ga. All sera were stored at −40 C and inactivated at 56 C for 30 min prior to testing.

The standard SN test (12) employed 70 to 100 mouse median lethal doses of standard rabies challenge virus and fivefold serum dilutions with TC medium as diluent. The test was performed with eight randomized male 10- to 12-g NYLAR mice per dilution. End points were determined by the method of Reed and Muench (14).

The tissue culture SN tests were performed with the same serum dilutions as were used in the mouse SN tests. From box titrations of hyperimmune antirabies serum of known mouse SN titer against tenfold dilutions of reconstituted ERA vaccine in tissue culture, the optimal challenge dose was determined to be a virus dilution of 10^{-1} ($10^{1.7}$ − $10^{2.5}$ MICLD$_{50}$/0.02 ml).

The dilutions of test sera were incubated at room temperature for 30 min with the constant virus challenge. After removal of the growth medium by suction, the BHK-21 cells were inoculated with 0.05 ml of each serum-virus dilution per chamber. The chambers were then filled with 0.5 ml of maintenance medium. After 5 days of incubation in a CO_2 incubator, the cells were examined for fluorescent rabies antigen. Interpretation of the test was made by observing the extent of fluorescent antigen present in cells exposed to the serum-virus dilutions. The highest serum-virus dilution producing nearly complete neutralization of fluorescence was considered the end-point titer. Since cells at any given virus-serum dilution showed either extensive or little if any specific fluorescence, interpretation was not difficult.

RESULTS

The BHK-21 cells formed confluent monolayers within 24 hr on the TC chamber slides, and intact cell sheets remained for the duration of the test.

Although fluorescent antigen was discernible as early as 36 hr after infection, the majority of the cells did not show extensive fluorescence until day 5. All SN tests, therefore, were read 5 days after serum-virus inoculation.

Table 1 presents a comparison of the rabies antibody titers of sera tested by the standard mouse SN test and the TC-fluorescent antibody (FA) procedure. The titers obtained by the TC-FA method with all but one of the 30 sera fell within a single fivefold dilution of that observed

TABLE 1. *Rabies antibody titers of sera tested in fivefold dilutions by serum neutralization mouse and tissue culture-fluorescent antibody (TC-FA) techniques*

Serum no.	Antibody titers	
	Mouse[a]	TC-FA[b]
Animal sera: 8112	<5	Negative
8129	<5	Negative
2B	<25	5
8125	12	5
8113	15	5
8114	11	5
8115	9	5
8116	7	5
8128	95	25
8122	34	25
2A	90	25
1B	79	25
1A1	315	125
1A3	1,849	625
1A5	1,002	625
SP2	1,253	625
DON	7,079	3,125
Human sera: 1730	<5	Negative
2016	<5	Negative
2017	<5	Negative
1725	9	5
1727	21	5
2018	8	5
2020	8	5
2022	10	5
2023	9	5
1833	5	5
1844	16	5
2019	5	5
2021	37	5

[a] Reciprocal of 50% serum neutralizing dilution.
[b] Reciprocal of the highest serum neutralizing dilution that inhibited rabies virus fluorescence.

by the mouse SN test. The one exception was a human serum found to have a TC-FA titer of 1:5, but a mouse SN titer of 1:37.

Repeated testing by the TC-FA method of duplicate samples of sera gave consistent results. A repeated mouse SN test gave results within the original fivefold dilution in all but two sera, although the calculated titers varied between tests. The two exceptions each decreased one fivefold dilution from the results of the first test.

DISCUSSION

After methods utilizing other virus-cell culture systems were studied, the ERA strain in BHK-21 cells was selected for determining rabies SN titers. A Flury high egg passage (HEP) strain of rabies virus (11, 15) provided by T. J. Wiktor, Wistar Institute, Philadelphia, Pa., was inoculated into BHK-21 and African green monkey (Vero) cells (19). This virus strain, received with a NYLAR suckling $MICLD_{50}$ titer of $10^{4.1}/0.02$ ml, infected both cell culture systems as demonstrated by the FAT, but a titer greater than $10^{2.5}$ $MICLD_{50}$ per 0.02 ml in three TC passages was not achieved, nor was any cytopathic effect (CPE) produced. However, after a single intracerebral suckling mouse passage of the HEP strain, CPE was produced in 7 to 9 days in Vero cells. However, the need for a high virus challenge (10^3 $MICLD_{50}/0.02$ ml) to obtain consistent CPE makes this virus strain less sensitive for a SN test.

The primary criterion for the selection of this system for the TC-FA procedure was the extensive fluorescence observed with ERA in BHK-21 cells.

Although the serum-virus neutralization test in mice has been the method of choice for determining rabies antibody titer in serum, the extensive care and facilities needed for the animals, as well as the length of time required for completion of the test, make the method less desirable. In comparison, the TC-FA procedure, although requiring TC facilities and a fluorescent microscope, takes only 5 days to complete.

The correlation of results by the TC-FA method with those of the mouse SN test, although a different virus and challenge dose are used, suggests its applicability in rabies research. The reproducibility of the test with serum titers ranging from $\frac{1}{5}$ to $\frac{1}{15,625}$ is a further indication of the reliability of the TC-FA procedure.

Determination of the end-point titer in the TC-FA method, using only one chamber serum-virus mixture, depends on the serum dilutions selected, whereas end-point titers in the mouse SN test are mathematically calculated between dilutions.

LITERATURE CITED

1. Abelseth, M. K. 1964. An attenuated rabies vaccine for domestic animals produced in tissue culture. Can. Vet. J. 5:279–286.

2. Abelseth, M. K. 1964. Propagation of rabies virus in pig kidney cell culture. Can. Vet. J. 5:84–87.

3. Debbie, J. G., and C. V. Trimarchi. 1970. Pantropism of rabies virus in free-ranging rabid red fox, *Vulpes fulva*. J. Wildl. Dis. 6:500–506.

4. Fernandes, M. V., T. J. Wiktor, and H. Koprowski. 1963. Mechanism of the cytopathic effect of rabies virus in tissue culture. Virology 21:128–131.

5. Fernandes, M. V., T. J. Wiktor, and H. Koprowski. 1964. Endosymbiotic relationship between animal viruses and host cells. A study of rabies virus in tissue culture. J. Exp. Med. 120:1099–1116.

6. Hronovský, V., R. Benda, and J. Činátl. 1968. Process of adaptation of street rabies virus to dog kidney cell primary cultures. Acta Virol. (Prague) Engl. Ed. 12:233–240.

7. Hummeler, K., H. Koprowski, and T. J. Wiktor. 1967. Structure and development of rabies virus in tissue culture. J. Virol. 1:152–170.

8. Johnson, H. N. 1964. Rabies virus, p. 356–380. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral and rickettsial diseases, 3rd. ed. American Public Health Assoc. Inc., New York.

9. Kaplan, M. M., T. J. Wiktor, R. F. Maes, J. B. Campbell, and H. Koprowski. 1967. Effect of polyions on the infectivity of rabies virus in tissue culture: construction of a single-cycle growth curve. J. Virol. 1:145–151.

10. Kondo, A. 1965. Growth characteristics of rabies virus in primary chick embryo cells. Virology 27:199–204.

11. Koprowski, H. 1954. Biological modification of rabies virus as a result of its adaptation to chicks and developing chick embryos. Bull. World Health Organ. 10:709–724.

12. Matsumoto, S. 1970. Rabies virus. Advan. Virus Res. 16:257–301.

13. Mikhailovsky, E. M., and M. A. Selimov. 1966. Adaptation of street rabies virus to primary Syrian hamster kidney cell culture. Acta Virol. (Prague) Engl. Ed. 10:373–379.

14. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. Amer. J. Hyg. 27:493–497.

15. Sedwick, W. D., and T. J. Wiktor. 1967. Reproducible plaquing system for rabies, lymphocytic choriomeningitis, and other ribonucleic acid viruses in BHK-21/13S agarose suspensions. J. Virol. 1:1224–1226.

16. Wiktor, T. J., M. V. Fernandes, and H. Koprowski. 1964. Cultivation of rabies virus in human diploid cell strain WI-38. J. Immunol. 93:353–366.

17. World Health Organization. 1966. Laboratory techniques in rabies 2nd ed., Monogr. Ser. 23. World Health Organization, Geneva.

18. Wiktor, T. J. 1966. Kinetics of rabies virus growth in tissue culture, p. 9–14. *In* Proceedings National Rabies Symposium, National Communicable Disease Center, Public Health Service, Atlanta, Georgia, May 1966.

19. Yasumura, Y., and Y. Kawakita. 1963. Study on SV₄₀ virus in tissue culture. Nippon Rinsho 21:1201–1206.