NOTES

Comparison Between a Modified Counterimmunoelectrophoresis Test and the Indirect Fluorescent-Antibody Test for Detection of Antibodies to Rabies Virus in Human Sera

ANA MARÍA DÍAZ* AND DONALD M. MYERS

Pan American Zoonoses Center, Pan American Health Organization, World Health Organization, Casilla 3092, Correo Central 1000, Buenos Aires, Argentina

Received 30 January 1981/Accepted 12 May 1981

The correlation between the modified counterimmunoelectrophoresis test and the indirect fluorescent-antibody test for detecting rabies virus antibodies in human sera was studied, using as a reference the standard mouse neutralization test. Higher sensitivity was observed with the counterimmunoelectrophoresis technique than with the indirect fluorescent-antibody method. The counterimmunoelectrophoresis test provides a practical quantitative alternative to the indirect fluorescent-antibody method.

The detection of rabies antibodies is of great value for epidemiological studies, determination of the immune status of man and animals, and diagnosis of the disease. Several serological procedures have been described for that purpose. Some of them measure neutralizing antibodies induced mainly by the viral glycoprotein, for example, the standard mouse neutralization (SN) (1), plaque reduction (15), rapid-fluorescent-focus-inhibition (12) and mixed hemadsorption (5) tests. Other techniques, such as the indirect fluorescent-antibody (IFA) test (8, 10, 14), complement fixation test (6), precipitation test (13), enzyme-linked immunosorbent assay (2), and indirect immunoassay (9), mainly measure antibodies to the ribonucleoprotein antigen.

This study was undertaken to evaluate the sensitivity and specificity of the modified counterimmunoelectrophoresis (CIE) (4) and the IFA tests (8, 10, 14) as compared with the SN test (1) for the detection of antibodies to rabies virus.

Recently, we developed a modified antigen-antibody-binding procedure incorporating the use of the CIE technique as an indicator system (4) and found that results correlated well with those of the SN test. This was due, in part, to the fact that both quantitative tests were shown mainly to measure immunoglobulin G (IgG) antibodies and to the fact that, because of the method used for its preparation and inactivation, the antigen was likely to recognize neutralizing antibodies. The correlation coefficient between

SN and CIE titers of sera from individuals under postexposure rabies immunization was found to be $r = 0.58$. In addition, the linear regression line we obtained when plotting the reciprocals of CIE and SN titers expressed as logarithms led us to conclude that serum SN titers could be estimated on the basis of CIE results.

The IFA test has been reported to be a sensitive qualitative method for detecting rabies antibodies and to correlate well with the SN test, although some differences have been observed (8, 14). Subsequent studies comparing these methods quantitatively have shown that antibody titers obtained by the IFA test did not correlate with the serum titers obtained in the SN test (3, 5, 11). The differences were attributed to the fact that the predominant antibody measured by the IFA test was probably directed toward viral ribonucleoprotein and is not considered to have neutralizing activity (9). It has also been noted that antibody detectable in the IFA test is more affected by adverse conditions, such as freezing and thawing or hemolysis, than are neutralizing antibodies (10).

We examined 89 human sera from a group of individuals comprising personnel and trainees of the Pan American Zoonoses Center (PAHO/WHO) and workers from other institutions who had received pre- or postexposure rabies treatment with suckling mouse brain rabies vaccine and whose sera were tested serologically in our laboratory.

The CIE tests were carried out basically as

described previously by Díaz and Myers (4). Briefly, the antigen was prepared from suckling rabbit brain infected with the rabbit-adapted challenge virus standard strain, as a 40% suspension in 0.01 M phosphate buffered-saline (pH 7.2), and was subsequently inactivated with beta propiolactone and centrifuged at $17,000 \times g$ for 10 min. The resulting supernatant fluid was removed and heated in a water bath at 56°C for 30 min to destroy hydrolytic enzyme activity.

An equine hyperimmune antirabies serum containing 200 IU/ml was used as indicator serum for CIE antibody titrations. To determine the optimal dilutions which produced a sharp precipitation band, the serum was diluted in phosphate-buffered saline and titrated against dilutions of the antigen.

The CIE test was conducted as follows. Equal volumes of the optimal antigen dilution and of test serum dilutions were incubated for 60 min at 37°C . Samples (8 ml each) of 0.9% agarose in 0.05 M Veronal buffer (pH 8.2) were poured onto each glass slide (7.5 by 5 cm). Wells 6 and 3 mm in diameter were cut to form two parallel rows 8 mm apart. The distance between wells was 10 mm.

At the end of the incubation period, the antigen-serum mixtures were placed in the cathodal wells (6 mm) of the slide and electrophoresed for 45 min at a potential difference of 10 V. The diluted hyperimmune equine serum was then placed in the corresponding anodal wells of the slide, and electrophoresis was continued for 120 min. At the end of the run, precipitation bands were detected in normal control sera and in sera with titers below 1:5. Sera with higher titers did not produce precipitation bands, thus indicating total antigen binding.

The SN test was carried out by intracerebral inoculation in mice as described by Atanasiu et al. (1). A titer of 1:5 or higher was considered to be positive.

The screening of sera for rabies antibodies by the IFA test was performed as reported by Larghi (7). Briefly, impression smears of mouse brains infected with street rabies virus were made on glass slides, dried, and fixed in cold acetone at -20°C for 4 h. Sera at a 1:5 dilution were incubated on the smears for 30 min at 37°C in a moist chamber. After the smears were washed, fluorescein isothiocyanate-conjugated rabbit antihuman globulin was applied for 30 min at 37°C . After being washed and mounted with glycerol-phosphate-buffered saline, specimens were examined with a Leitz ultraviolet microscope equipped with UG-1 exciting filter and an ultraviolet-excluding barrier filter.

The immunoglobulins detected by IFA were identified by means of rabbit anti-human sera specific for IgM, IgA, and IgG.

The criteria for grading fluorescence were sim-

ilar to those adopted by Leffingwell and Irons (10): +++++ = numerous bright fluorescing bodies; +++ = slight reduction in intensity of fluorescence and in number of fluorescing bodies; ++ and + = great reduction in intensity of fluorescence and in number of fluorescing bodies; - = no fluorescence.

Table 1 shows the results obtained with the three test methods. There was complete agreement between the CIE and IFA tests in 60 of the 76 SN positive sera (SN titers ranging from 1:5 to 1:3,125 or greater). In addition, 14 serum samples positive by SN and CIE tests gave negative results when screened by the IFA test. Agreement in sensitivity between CIE and SN positive sera was 97.3%, whereas agreement with the IFA test was 80.2%. When the data resulting from the CIE and SN tests were subjected to statistical analysis, a correlation coefficient of $r = 0.60$ was obtained. This correlation factor is identical to that obtained earlier (4).

It was of interest to note that most of the 14 sera found positive by SN and CIE and negative by IFA were from persons who had been vaccinated 2 years earlier. The other persons had been immunized 1 to 9 years before.

The discrepancy in the results obtained with the different tests led us to determine the class of immunoglobulins present in the sera of which an adequate volume was available: seven early sera (10 days) and seven late sera (2 to 5 years). The rabies specific antibodies detected by the IFA tests with the early sera were found to be IgG, IgM, and IgA, whereas in late sera, activity was restricted primarily to IgG antibodies (Table 2).

All of the 10 serum samples found to be negative for SN antibodies were also negative by both CIE and IFA tests (specificity 100%).

In conclusion, the CIE test provides an alternative method to the IFA and SN tests for the detection of serum antibodies to rabies virus. The performance of the CIE system is technically simple and requires approximately the same amount of time as does the IFA test. In this study, the CIE test was found to be more

TABLE 1. Correlation among results of SN, CIE, and IFA tests on sera from individuals under pre- and postexposure treatment for rabies

Range of SN titers*	No. of samples with following results			
	CIE+/IFA+	CIE+/IFA-	CIE-/IFA+	CIE-/IFA-
<5	0	0	0	10
5-25	9	1	1	1
26-125	14	7	0	0
126-625	11	3	0	0
626-3,125	24	3	0	0
>3,125	2	0	0	0

* Titers expressed as reciprocals of serum dilution.

TABLE 2. *Immunoglobulins detected by IFA and titers obtained by CIE and SN tests in sera from 14 individuals vaccinated against rabies*

Group	Serum no.	Immunoglobulins detected by IFA ^a			Titer ^b	
		IgM	IgG	IgA	CIE	SN
Early bleeding (10 days)	1	—	++++	—	40	3,125
	2	+	++++	+	40	37
	3	+	++++	+	20	37
	4	+++	+	—	20	5
	5	+	++++	+	10	5
	6	—	+	—	8	47
	7	+	++++	+	10	30
Late bleeding (2 to 5 years)	1	—	+	—	5	66
	2	—	+	—	5	66
	3	—	+	—	5	280
	4	—	+	—	25	625
	5	—	+	—	10	84
	6	—	++	+	24	3,125
	7	+	++	+	44	625

^a Degree of fluorescence graded + to +++++.^b Titers expressed as reciprocals of serum dilution.

sensitive than the IFA test for determining the immune status of individuals who had received rabies immunization at some time in the past. The sensitivity of the CIE technique was comparable to that of the SN test. Furthermore, CIE permits the quantitative determination of titers of rabies serum antibodies in approximately 4 h, whereas the SN test requires 15 days. It shares the advantage with other rapid tests (2, 4, 12) of providing the physician with information on the immune status of a patient after rabies exposure and during postexposure treatment.

Appreciation is expressed to Graciela Perdomo and Oscar Becco for their efficient technical assistance.

LITERATURE CITED

- Atanasiu, P., M. Bahmayar, M. Baltazard, A. Komarov, H. Koprowski, P. Lepine, F. Pérez-Gallardo, and M. Schaeffer. 1956. Rabies neutralizing antibodies response to different schedules of serum and vaccine inoculations in non-exposed persons. *Bull. W.H.O.* 14:593-611.
- Atanasiu, P., V. Savy, and P. Perrin. 1977. Epreuve immunoenzymatique pour la détection rapide des anticorps antirabiques. *Ann. Microbiol. (Paris)* A128:489-498.
- Campolina Diniz, C. C., and R. Reis. 1975. Comparative studies between indirect immunofluorescence and serum neutralization procedures in rabies antibodies titration of vaccinated cattle. *Arq. Esc. Vet. Univ. Fed. Minas Gerais* 27:29-37.
- Diaz, A. M., and D. M. Myers. 1980. Determination of serum neutralization antibodies to rabies virus by a modified counterimmunoelectrophoresis test. *J. Clin. Microbiol.* 12:175-179.
- Grandien, M. 1977. Evaluation of tests for rabies antibody and analysis of serum responses after administration of three different types of rabies vaccines. *J. Clin. Microbiol.* 5:263-267.
- Kuwert, E. 1973. The complement fixation test, p. 124-134. In M. M. Kaplan and H. Koprowski (ed.), *Laboratory techniques in rabies*, 3rd. ed. World Health Organization (Monograph Series 23), Geneva.
- Larghi, O. P. 1975. Prueba de anticuerpos fluorescentes para rabia. Centro Panamericano de Zoonosis OPS/OMS (Nota Técnica 8). Ramos Mejía.
- Larsh, S. E. 1965. Indirect fluorescent antibody and serum neutralization response in pre-exposure prophylaxis against rabies. *Ann. Intern. Med.* 63:955-964.
- Lee, T.-K., H. D. Hutchinson, and D. W. Ziegler. 1977. Comparison of rabies humoral antibody titers in rabbits and humans by indirect radioimmunoassay, rapid-fluorescent-focus-inhibition technique, and indirect fluorescent-antibody assay. *J. Clin. Microbiol.* 5:320-325.
- Leffingwell, L., and J. V. Irons. 1965. Rabies antibodies in human serums titrated by the indirect FA method. *Public Health Rep.* 80:999-1004.
- Peck, F. B., Jr. 1966. The detection of human rabies antibody by the indirect fluorescence test. *Symp. Ser. Immunobiol. Stand.* 1:201-206.
- Smith, J. S., P. A. Jager, and G. M. Baer. 1973. A rapid reproducible test for determining rabies neutralizing antibody. *Bull. W.H.O.* 48:535-541.
- Sokol, F., H. D. Schlumberger, T. J. Wiktor, and H. Koprowski. 1969. Biochemical and biophysical studies on the nucleocapsid and on the RNA of rabies virus. *Virology* 38:651-665.
- Thomas, J. B., R. K. Sikes, and A. S. Ricker. 1963. Evaluation of indirect fluorescent antibody technique for detection of rabies antibody in human sera. *J. Immunol.* 91:721-723.
- Wiktor, T. J. 1973. Tissue culture methods, p. 113-115. In M. M. Kaplan and H. Koprowski (ed.), *Laboratory techniques in rabies*, 3rd. ed. World Health Organization (Monograph Series 23), Geneva.