Serodiagnosis of Rabies by Dot Immunobinding Assay

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A dot immunobinding assay that uses inactivated antigen for the detection of rabies viral antibodies was compared with the rapid fluorescent focus inhibition test. Results of testing pre- and postvaccination sera from humans ($n = 33$) and canines ($n = 22$) were identical for both tests. Endpoint titers of positive sera also were approximately the same by both methods. When a mouse monoclonal antibody was used, the dot immunobinding assay antigen was shown to possess detectable rabies virus glycoprotein and core antigens.

Although the diagnosis of rabies is generally accomplished by detecting the virus in tissues or body fluids, there is a need for a reliable and reproducible serological test that may be used in animal population surveys, evaluation of vaccines, and prophylaxis, as well as for supplementing antigen detection. The procedure favored by most laboratories for such determinations is a tissue culture neutralization test using live rabies virus in an immunofluorescent-antibody staining technique (rapid fluorescent focus inhibition test [RFFIT]) developed by Smith et al. (3). The recent successful application of a rapid, sensitive, and specific dot immunobinding assay (DIA) (2) for routine detection of antibodies to various viruses suggested its use for assaying rabies viral antibody. The DIA is simple to perform, requires little in the way of laboratory equipment, uses an inactivated viral antigen, and is cost effective. This report provides a comparison of the DIA with the standard procedure (RFFIT) as applied at the Centers for Disease Control for the determination of rabies viral antibody.

MATERIALS AND METHODS

Test specimens. A total of 33 human sera (17 from negative, unvaccinated individuals and 16 from positive vaccinees) and 22 canine sera (3 from positive vaccinees and 19 controls) were included in this study. These specimens had all been assayed previously by RFFIT (3) at the Centers for Disease Control and were examined by DIA by examiners without previous knowledge of the results. Sera were diluted 1:4 in maintenance medium without fetal bovine serum, which had been recovered from uninfected cell cultures (control antigen, see below). This was done to block non-specific reactivity. Immune mouse ascites fluids containing monoclonal antibody to rabies viral glycoprotein or N protein were also included.

Test antigens. The rabies virus in this study is a vaccine strain that was provided by the Veterinary Department, Merieux Laboratories, Inc., Athens, Ga. The virus is a Pitman-Moore strain of fixed rabies virus derived from the original Louis Pasteur isolate of 1882 and maintained by the National Institutes of Health under reference NIH PV-11. It has been passaged in various cells and cell lines and most recently was adapted to a hamster embryo cell line (Nil₂). The 48-h-postinfection virus harvest titer of $10^{7.7}$ tissue

culture infective doses per ml was inactivated with β -propiolactone, and 0.001% thimerosal was added as a preservative. The virus was aspirated from the infected hamster cells and, except for filtration through a 1- μ m-pore-size filter, not further purified before use. Control antigen, consisting of serum-free culture medium removed from uninfected hamster embryo cells, was the same preparation used as serum diluent. The medium for virus production was also serum free, although fetal bovine serum was in the cell growth medium.

Test procedures. Both the neutralization test and the DIA have been described previously (2,3) and essentially were done as follows.

The RFFIT as used at the Centers for Disease Control consists of mixing dilutions of test serum in cell culture medium with a constant amount of rabies virus in a multichambered cell culture slide. Serum and virus are then incubated at 37°C for 90 min. BHK-21/13S cells are added to each well, and incubation at 37°C is continued for another 24 h. The cultures are then rinsed, fixed in acetone, and stained with fluorescein-conjugated, horse anti-rabies virus antiserum. A serum is considered to have antibody when 50% or more of the cells are protected from the virus.

The DIA as used in this study consisted of dotting undiluted antigen on nitrocellulose sheets (BA85/21SD, 0.45 μ m pore size; Schleicher & Schuell, Inc., Keene, N.H.) with an imprinted 3-mm grid. Sheets with grids are more convenient to use because they provide a simple mechanism for spacing the antigens and for maintaining a record of each test. Viral and control antigens (1 μ l each) are spotted in adjacent squares with two empty squares between pairs. The sheets are allowed to dry at room temperature for approximately 0.5 h. The sheets are then submerged in BLOTTO (5% nonfat dry milk in phosphate-buffered saline [PBS] [pH 7.2] containing 0.01% Antifoam A [Sigma Chemical Co., St. Louis, Mo.] and 0.001% thimerosal) to block the unreacted protein-binding sites. This BLOTTO is rinsed off after 1 h with PBS-Tween 20 (0.1%; Sigma), and the sheets are air dried. The sheets may be used at this time or stored at -20°C for future use. No detectable loss of activity has been noted with -20°C storage for as long as 12 months.

To test for antibody, the nitrocellulose sheets are placed on blotting paper (no. 470; Schleicher & Schuell) saturated with PBS-Tween to keep them moist. Sheets of the size used in these tests fit comfortably in square plastic dishes (100 by 15 mm, Integrid petri dish; Becton Dickinson Labware,

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TABLE 1. Comparison of the RFFIT and DIA for rabies viral antibody detection in human and canine sera

Sera	No. positive/total by:		No. of sera providing correct test results ^a
	RFFIT	DIA	
Human control sera	0/17	0/17	17
Human vaccinee sera	16/16	16/16	16
Canine sera	3 ^b /22	3 ^b /22	22

^a Concordance of RFFIT and DIA results was 100% for all types of sera tested.

^b Positive sera were from the three vaccinated dogs; the other 19 sera were from unvaccinated control dogs.

Oxnard, Calif.). Test sera previously diluted in control antigen are applied by saturating absorbent paper strips (3 by 6 mm) or disks (6 mm diameter) (lintless paper strips; Gelman Sciences, Inc., Ann Arbor, Mich.) and placing them over the test antigen dots. Contact between adjacent antibody-containing strips or disks must be avoided. The sheets are then placed in a humidified incubator for 1 h at 37°C, submerged in PBS-Tween for three 5-min washes with shaking, and then submerged in 10 ml of goat anti-human, canine, or mouse immunoglobulin G conjugated with alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) diluted 1:1,000 in PBS. For testing of other animal species, the appropriate anti-immunoglobulin conjugates must be used, or staphylococcal protein A-enzyme conjugate and appropriate substrate may be substituted. After 1 h of incubation at 37°C, test sheets are again washed three times for 5 min each with PBS-Tween and then submerged in substrate. The substrate used for the alkaline phosphatase conjugate was naphthol AS-MX phosphate with Fast Red TR (Sigma) (2). A red spot results at the site of antigen-antibody reaction. The reaction time was that required for the color development of the positive control, which usually occurred in 5 to 15 min. The substrate is then washed off with tap water, and the nitrocellulose sheets are allowed to dry at room temperature.

Undiluted viral antigen preparation was selected for use by screening 1:4 dilutions of positive and negative sera against twofold dilutions of antigen. Undiluted antigen yielded the most intense reaction and allowed the easiest discrimination between positive and negative sera. A positive response is defined as a 1+ color intensity, with 4+ as a maximum. Reproducibility of color intensity between tests is controlled by stopping the reaction each time when the positive control is at a predetermined intensity. Overdevelopment tends to cause nonspecific responses to control antigen. This also was noted frequently if the sera were not diluted in control culture fluid.

RESULTS

The results of testing human sera from normal (pre-vaccination) and rabies virus-vaccinated individuals are shown in Table 1. The DIA showed 100% specificity and sensitivity compared with the RFFIT (16 of 33 positive in both cases). In preliminary studies, sera were diluted in PBS. However, considerable nonspecific staining was noted in the vaccinee sera. This was eliminated by diluting the sera in the control antigen before testing. Of 22 canine sera, 3 were positive by both tests; these were from vaccinated dogs.

When selected sera were titrated to 50% endpoint by the RFFIT and to a 1+ reaction intensity by the DIA, similar titers were obtained (Table 2). Additionally, the intensity of

red color detected in the DIA with positive sera was proportionately greater as the RFFIT titers were increased. This allows a subjective estimate of the serum antibody titers when testing a single dilution, usually 1:4. An important use for a rabies antibody assay is to test for response to vaccination. Since antibody to viral glycoprotein antigen (neutralizing antibody) is necessary to provide protection, it was desirable to demonstrate that the DIA antigen contained detectable glycoprotein. This was done with immune mouse ascites fluid containing monoclonal antibody to rabies viral glycoprotein. When used in the DIA, this immune mouse ascites fluid had a titer of 1:2,560, showing a high degree of reactivity with the antigen being used in these studies. Immune mouse ascites fluid containing monoclonal antibodies reactive with rabies virus N protein also reacted strongly with this antigen.

DISCUSSION

Rabies is generally diagnosed by the detection of virus in tissue specimens rather than by determining the development of antibody in response to infection. However, antibody does result after infection or vaccination, and as a consequence serological procedures are required. Several serological methods are currently available and may be used for diagnostic purposes (1, 3). A DIA recently reported by us (2) has been found to be rapid as well as specific and sensitive for the detection of antibody and the identification of virus antigen. In addition, the simplicity of the procedure permits its performance under field conditions, requiring little in the way of equipment. The procedure is also relatively inexpensive.

Although the number of human rabies cases occurring in the United States per year is relatively small, exposure to possibly rabid wildlife requires that thousands of individuals each year receive prophylactic treatment. The availability of a rapid, simple test with inactivated viral antigen for rabies virus antibody, such as that described herein, would allow evaluation of the success of this prophylaxis in most laboratories, including those in underdeveloped areas. In addition, the DIA might be used in the field for surveillance of animal populations, in the evaluation of vaccine efficacy, and for optimizing immunization schedules.

The data obtained in this study show a good correlation between the RFFIT and DIA results for determining the antibody status of vaccinated humans and dogs. However, the use of appropriate control reagents and dilutions of sera in medium from control cell cultures is important, because

TABLE 2. Comparison of RFFIT and DIA endpoint rabies viral antibody titers in human sera

Serum no.	Titer	
	RFFIT	DIA (color intensity rating)
1	1:280	1:160 (3+) ^a
2	1:125	1:160 (2+)
3	1:280	1:160 (4+)
4	1:56	1:40 (1+)
5	1:250	1:160 (2+)
6	1:700	1:160 (3+)
7	1:56	1:10 (1+)
8	1:29	1:160 (2+)
9	1:9	1:40 (2+)
10	1:17	1:40 (2+)

^a Relative intensity of red detected in the antigen-antibody dot of a serum dilution of 1:4.

nonspecific reactions were noted in sera from vaccinees. The observation of nonspecific staining in these sera is most likely a result of cell culture antigens in the rabies vaccine, inasmuch as control antigen blocks this reaction.

In general, the sensitivity of the DIA was comparable to that of the RFFIT by virtue of the similar endpoint titers for rabies virus obtained by both procedures. It also is clear that neutralizing (glycoprotein) antibodies can be detected by the DIA, in addition to those directed against other viral proteins. Modification of the test would be necessary to enable the specific assay of glycoprotein antibodies.

Additional studies are required to determine the potential usefulness of the DIA for detecting antibody in encephalitis patient sera, as well as viral antigens in fluids (serum, cerebrospinal fluid, tears) and tissues of patients. It seems clear, however, that the DIA is useful as a simple, specific, and sensitive test for the determination of the status of rabies vaccinees, including humans and canines, as well as for surveying animal populations for the occurrence of rabies infections. This could include a variety of species if a protein A conjugate were used for the detection of immunoglobulin

G. In this way, species-specific anti-immunoglobulins would not be required. Further study is required to determine the feasibility of this approach.

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