Rapid Test for Detection of Rabies Antibodies in Human Serum

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A simple, sensitive, rapid method based on the principle of immunoadherence hemagglutination (IAHA) has been devised for the detection of rabies antibody. In this test, fixation of complement to complexes of rabies antigen with specific antibodies is readily detected by agglutination of human erythrocytes bearing receptors for C3. Sera from individuals undergoing preexposure rabies immunization were tested for rabies antibodies by the IAHA method and by a virus neutralization test performed in tissue culture, the rapid fluorescent focus inhibition test. IAHA titers showed a high degree of correlation with rapid fluorescent focus inhibition test titers, although it is not known whether results of the IAHA test represent the detection of neutralizing antibodies. An advantage of the IAHA test over the rapid fluorescent focus inhibition test was that results were obtained in a shorter period of time. In some instances, this can be of clinical significance in determining antibody levels to rabies virus. Furthermore, the IAHA test is most applicable as a rapid screening tool for the detection and quantitation of rabies antibodies in vaccinated subjects.

The standard procedure for measuring rabies antibodies in serum is based on the inhibition of rabies infection in animals or tissue culture (1, 3, 9, 10). This procedure is time consuming, costly, and impractical for use in a routine virus laboratory. There is a need for a simple, low-cost, rapid test for the detection of rabies antibody in human and animal sera.

In this paper, we report the adaptation of the immune adherence hemagglutination (IAHA) test for use in the rapid detection and quantitation of rabies antibodies in human serum.

MATERIALS AND METHODS

Sera. The sera used in this study were obtained from the following three sources: (i) volunteers who had received preexposure immunization with rabies vaccine (2), (ii) individuals being screened for rubella antibody, and (iii) sick or recently sick persons with a suspected viral infection other than rubella or rabies. Rabies vaccine recipients had participated in the clinical trial of a new rabies vaccine derived from rhesus diploid cells. Each participant in the vaccine study received at least two injections of vaccine. Serum was obtained from each participant before the first immunization, 2 weeks after the first injection, and 2 weeks after administration of the second dose. Sera from individuals in this group had been tested previously for rabies antibody by the rapid fluorescent focus inhibition test (RFFIT). Vaccination sera and sera with low, moderate, and high levels of rabies-neutralizing antibody were selected for IAHA testing. These sera were coded before submission to the laboratory for IAHA testing.

The sera screened for rubella antibody were derived primarily from women. These sera, submitted to the Michigan Department of Public Health Virus Laboratory, Lansing, to determine immune status to rubella virus, were tested for rabies antibody by the IAHA method.

The sera derived from sick or recently sick individuals had been submitted to the Virology Laboratory for the purpose of confirming a viral infection. An acute- and convalescent-phase serum was submitted on each of these individuals. These sera were tested by the IAHA method with the following antigens: influenza A, Mycoplasma pneumoniae, cytomegalovirus, herpes simplex virus, and Chlamydia group antigen. A fourfold or greater antibody response against at least one of these viral antigens was detected in each of these individuals.

Rabies antigens. Baby hamster kidney (BHK-21) cells were used for both the propagation of the virus and the preparation of the rabies antigen. The cells were maintained in Eagle minimal essential medium supplemented with 5% heat-inactivated fetal calf serum, buffered with sodium bicarbonate (1 g/liter), and containing penicillin (50 U/ml) and streptomycin (50 μg/ml). Rabies virus CVS-11 (6) was used to prepare the antigen. When the cells were infected with the virus, the medium contained no fetal calf serum. Virus was added to cells at a multiplicity of infection of 0.5 to 1.0, and the virus-cell mixture was incubated at 37°C until a 90% cytopathic effect was observed (generally 48 h after inoculation). Cellular debris was removed by centrifugation at 2,000 rpm (450 × g) for 10 min at room temperature. The cell-free supernatant fluid was chilled to 4°C and filtered through a membrane filter (0.45 pore size; Millipore Corp., Bedford, Mass.).
Virus was inactivated by treatment with β-propiolactone (0.25%) at 4°C for 18 h. Residual β-propiolactone was hydrolyzed by incubation for 2 h at 37°C. Samples of the antigen solution were stored at −70°C until used. Antigen preparations were found to maintain their titers for at least 1 year. The antigen titer was determined in block titration against a standardized rabies antiserum. The endpoint titer of the antigen measured by the immune adherence test was in the range of 1:4 to 1:8. Antigen controls consisting of noninfected cell cultures were included in each IAHA test.  

**RFFIT.** The RFFIT was performed by the method of Smith et al. (10). Each serum was inactivated at 56°C for 30 min before inclusion in the test and then serially diluted fivefold after an initial 1:5 dilution. The challenge rabies virus was added and incubated for 1 h at 37°C in a controlled-humidity, 5% carbon dioxide chamber. After incubation, the serum-virus mixtures were put into Lab-Tec 8 chamber slides (Miles Laboratories, Inc., Naperville, Ill.), and BHK-2 cells were added at 10² cells per well. The slides were returned to the 5% carbon dioxide chamber and incubated for 24 h at 37°C. After incubation, the growth medium was removed, and the slides were rinsed in phosphate-buffered saline and once in acetone at room temperature and fixed for 1 min in acetone at −20°C. Slides were dried, stained with fluorescein conjugate, and observed. Titers were expressed as the reciprocal of the highest serum dilution showing a 50% reduction in the number of virus foci with respect to cultures containing virus but not antiserum. An initial one- to fivefold dilution was made of each serum.

**IAHA.** The IAHA method described by Lennette and Lennette (8), with slight modifications, was used to determine rabies antibody titers. Briefly, equal volumes (0.025 ml) of serial dilutions of serum and rabies antigen (1 U) were incubated in 96-well plastic plates (Linbro Scientific, Inc., Hamden, Conn.) at 37°C for exactly 35 min. After adding 50 μl of a 1:100 dilution of fresh guinea pig serum, the mixture was further incubated at 37°C for 45 min. Fifty microliters of a mixture of human type O erythrocyte suspension in dithiothreitol-EDTA-Veronal buffer was then added (8), and the plates were allowed to stand at room temperature for 1 to 2 h. The hemagglutination pattern was read, and titers were recorded as the reciprocal of the highest serum dilution showing positive agglutination. The titrations were started at a serum dilution of 1:4.

**RESULTS**

**Reproducibility of the IAHA test.** Ten sera were selected to confirm the reproducibility of the rabies IAHA procedure. Eight of these sera had demonstrable rabies antibody by the RFFIT. The titers ranged from 1:64 to 1:2,048. Of the 10 sera, 2 were without demonstrable rabies antibody at a dilution of 1:4. Ten replicate samples of each of these sera were tested on 10 different days to confirm reproducibility. The data obtained by the IAHA procedure were analyzed by the nonparametric method of Friedman as described by Lehmann (7). The IAHA rabies antibody test was found to be highly reproducible. The model, or expected antibody value, was observed 59% of the time. The fold difference, determined by a twofold serial dilution system, with respect to the model value was −2 for 18 sera, −1 for 162 sera, 0 for 479 sera, +1 for 130 sera, and +2 for 11 sera. Of the 800 observations (only sera with antibody titer were used for calculations), 96.4% were within one dilution of the model value.

**Specificity of the IAHA test.** Rabies antibody was not detected by the IAHA test in any of the 100 sera that had been submitted to the virus laboratory for rubella antibody testing, nor was any rabies antibody detected by the IAHA test in any of the 21 paired sera that had been submitted to the virus laboratory from individuals with a suspected or frank viral infection other than rabies.

Of the 21 individuals with suspected viral infection, 3 had fourfold influenza antibody rises, 3 had fourfold cytomegalovirus antibody rises, 7 had fourfold or greater antibody rises to herpes simplex virus, 3 had fourfold or greater rises to *Chlamydia* group antigen, and 5 had fourfold or greater rises to *M. pneumoniae*. The majority of the sera of these 21 individuals contained antibody against more than one viral antigen.

No demonstrable rabies antibody was found in any of 136 prevaccination sera obtained from the volunteers participating in the rabies vaccine trial. None of these 136 individuals had a previous vaccine vaccination history.

**Sensitivity of the IAHA test.** A total of 253 sera derived from volunteers who had participated in rabies vaccine clinical trials were tested for rabies antibody by the RFFIT and the IAHA method. Of these 253 sera, 136 were obtained before vaccination, and the other 117 were obtained after the two-dose vaccination series. No rabies antibody was detected by either the IAHA test or the RFFIT in the 136 prevaccination sera. Rabies antibody was detected by the RFFIT in all of the 117 postvaccination sera and by the IAHA test in 107 of these 117 sera. All of the sera with detectable IAHA rabies antibody were rabies antibody positive by the RFFIT. The 10 sera that were RFFIT positive and IAHA negative contained low levels of RFFIT antibody. Six of these sera had less than 1 IU, and the other four contained 1.3, 4.3, 3.1, and 1.9 IU of rabies antibody.

The IAHA and RFFIT positive and negative antibody values for these 253 sera were tested for relatedness by chi-square analysis, using a 2 × 2 table. All 107 sera positive by IAHA were also positive by RFFIT, whereas of 146 sera negative by IAHA, 10 were positive and 136 were negative by RFFIT. The chi-square value
was calculated as 216.5, with a $P$ value of $<0.005$.

Correlation between the IAHA and RFFIT procedures. Quantitative rabies antibody data were obtained by both the IAHA and RFFIT procedures on 107 sera from the vaccinated individuals. The quantitative antibody value from each test was transformed to the log base 2, and the RFFIT value was then plotted against its respective IAHA value. Correlation analysis of these data yielded a coefficient of correlation of 0.78, with an $R^2$ of 0.61, suggesting a significant correlation between the two tests (Fig. 1).

**DISCUSSION**

A number of in vitro rabies antibody tests in which standard serological methods are used have been reported previously (1, 9, 10). However, none of these procedures are practical or reliable for routine use in serology or virology laboratories. The IAHA test, a modification of the complement fixation test (4), is simple to perform. It does not require sophisticated equipment, and results can be obtained in approximately 4 h. We found the complement fixation test to be undersensitive when compared with the IAHA or the RFFIT procedures (unpublished data). The complement fixation test required the use of concentrations of rabies antigen which were 200- to 250-fold greater than those used in the IAHA test. The infection inhibition tests require the availability and maintenance of tissue cultures or animals and the use of live rabies virus, which many laboratories are not prepared to handle. Live rabies virus is not required for the IAHA procedure, and lot-to-lot variations of baby hamster kidney cell-derived rabies antigen is minimal. β-Propiolactone inactivation does not result in significant denaturation of the rabies antigen, and the antigen is stable in aqueous solution. We are currently attempting to establish whether the antigen can be freeze-dried without significant loss in potency. The results of these studies will be reported at a later date.

Our experience so far would indicate that there is less test-to-test variation with the IAHA test than with the RFFIT. The RFFIT appears to be more sensitive than the IAHA test in detecting small amounts of rabies antibody. This may reflect the inability of the IAHA procedure to detect low-affinity antibody (5).

If the level of rabies neutralizing antibody correlates with protection and if the complement-binding antibody correlates with neutralizing antibody, then the presence of moderate levels of IAHA rabies antibodies should equate with protection against rabies. Since the IAHA test relies on complement activation, both immunoglobulin M and immunoglobulin G are likely to be detected by it. With one exception, all sera with an IAHA rabies antibody titer of 1:32 or greater contained ≥1 IU of neutralizing antibody. It is our belief that the IAHA procedure offers an excellent practical tool for screening sera for rabies antibody. Our data would indicate that individuals with an IAHA rabies antibody titer of ≥1:32 should not require rabies vaccine. We are initiating protection studies in animals to confirm the relationship between the rabies antibody detected by the IAHA test and protection against rabies infection.

One consideration possibly weighing in the suggestion made from the Centers for Disease Control, Atlanta, Ga. that postimmunization to rabies vaccination not be routinely determined is the cost and involvement of the RFFIT. Such determinations might be encouraged by the relative ease and low cost of the IAHA test.

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**LITERATURE CITED**

