Evaluation of Tests for Rabies Antibody and Analysis of Serum Responses After Administration of Three Different Types of Rabies Vaccines

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Humoral antibody response to three types of rabies vaccines were assayed by the neutralization (NT), the mixed hemadsorption (MH), and the indirect immunofluorescence (IF) tests. The NT and MH tests were used to detect antibodies combining with antigens at the surface of virions and infected cells, whereas the indirect IF test measured antibodies mainly to the rabies nucleocapsid antigen. After immunization with a human diploid cell vaccine, antibodies were detected by both the NT and the MH test in the 14th- and 30th-day serum samples from each of eight vaccinated persons. There was a good correlation between titers obtained with the two tests in this group of vaccinees. Antibodies elicited by duck embryo and nervous tissue vaccines occurred less frequently and in lower titers. In these groups of vaccinees, 5 of 14 and 5 of 10, respectively, had antibodies detectable by the NT test in the 14th- and 30th-day sera but were negative by the MH test. It is suggested that this was due to the high levels of immunoglobulin M antibodies, which are known to be elicited by daily injections of vaccine. Since antibodies of the immunoglobulin M class are considered to be less important for protection against rabies, the MH test is recommended for immunity determinations. Compared with the NT test, this test also offers the advantage of being technically more convenient because of its capacity for testing numerous sera in a single run. Antibody titers obtained by the indirect IF test in the human diploid cell vaccine group were relatively low. Titers in the duck embryo and nervous tissue vaccine groups were higher but did not correlate with the results of the NT test.

The rabies antibody responses induced in humans by vaccines derived from nervous tissue and duck embryo have been thoroughly investigated in the past. The recently introduced vaccine prepared in human diploid cells is reported to be highly immunogenic (1, 15). The verification of an antibody response after immunization to rabies is of great importance, and is recommended by the World Health Organization (WHO) Expert Committee on Rabies 1973 (21).

Rabies serum antibody levels have previously been determined mainly by the neutralization (NT) test. Until recently, this time-consuming test was performed in mice, although during the last decade other techniques, exploiting cell culture systems, have been reported (4, 6, 17, 19). A hemagglutination inhibition (HI) test (12) and a passive hemagglutination test (7) have been described, which probably also measure antibodies directed against antigens on the surface of the virion. Antibodies against mainly nucleocapsid antigens have been assayed by the indirect immunofluorescence (IF) (11, 14, 22) and the complement fixation (13) tests. The technique of mixed hemadsorption (MH) (10) has been shown to be very sensitive in detecting antibodies to several viral and cell surface antigens on monolayer cultures. Estimation of antibody levels in sera of rabies-vaccinated animals with this method was first described by Espmark et al. (9).

The aim of this study was to assess the MH test for estimation of human antibodies to rabies and to compare the results with those obtained by the NT and indirect IF methods. For this purpose the antibody response after vaccination with three types of rabies vaccine was studied.

MATERIALS AND METHODS

Vaccination. Three types of commercially available vaccines were used. Recommended dosage schedules were followed.

HDCV. The human diploid cell vaccine (HDCV; Institut Mérieux, Lyon, France; vaccin rabique in-
active Mérieux) was prepared in tissue cultures of human diploid cells (WI-38) and inactivated with β-propiolactone. One milliliter of vaccine was given subcutaneously on days 0, 3, 7, and 21.

DEV. Duck embryo vaccine (DEV; Lilly Research Laboratories, Indianapolis, Ind.) was given to patients in daily doses of 1 ml of vaccine subcutaneously for 14 days.

Neutral tissue vaccine (NTV). Patients were given different types of killed rabies vaccine, produced in adult or suckling animals. Vaccines were given subcutaneously in daily doses for 8 to 14 days.

Sera. Humans, vaccinated after suspected exposure to rabies, were examined serologically. Sera were drawn from 21 vaccinees before and on about days 14 and 30 after the beginning of immunization.

Virus and cells. Virus strain. Flurry HEP rabies virus was passaged 16 times in a human diploid cell strain; the clone was purified in BHK-21/13S cells and passaged in BHK-21 cells. This strain was kindly supplied by T. Wiktor and H. Koprowski of the Wistar Institute, Philadelphia, Pa.

Cell cultures. The BS-C1 grivet monkey cell line was used throughout the work.

Serological tests. (i) NT. This test was performed as described by Debbie et al. (6). Preincubated virus-serum mixtures were inoculated into Leighton tubes containing cell monolayers on cover slips. After 5 days of incubation, the monolayers were examined by immunofluorescence for the presence of rabies antigen.

All tests were performed with 30 to 300 tissue culture infective doses (TCID₅₀) of rabies virus. Serial 10-fold dilutions of the virus were made in the presence of inactivated negative horse serum to stabilize the virus. Human sera were tested in fourfold dilutions starting from 1:6.25. Two culture tubes were inoculated with each virus-serum dilution mixture. The final serum dilution yielding 50% negative tubes was used to express serum titers, which were then converted into international units (21).

WHO First International Standard freeze-dried natural anti-rabies horse serum (1955), containing 86.6 IU/ampoule reconstituted to 1 ml, was used as a reference, and anti-rabies serum of equine origin (Lederle), containing 1,000 IU/vial (lot no. 246-394), was used as control serum and included in all tests.

(ii) MH test. Tests were performed in milk dilution bottles with monolayers of cells infected with 5.5 × 10³ TCID₅₀ of rabies virus per bottle and then were incubated for 3 days at 37°C. At this time no cytopathic changes could be seen, but immunofluorescence showed cytoplasmic inclusions in all cells. From filter paper disks soaked with undiluted test serum, the antibodies were allowed to diffuse during 48 h from the top of an agar layer to the monolayer of either virus-infected or uninfected control cells. After removing the agar layer, antibodies combined with the surface of the virus-infected cells were demonstrated by sheep erythrocytes coated with globulin and bound to the antibodies via an antoglobulin link (8). Circular zones of hemadsorption were obtained if antibodies in test serum had combined with the surface of the virus-infected cells in the monolayer.

The diameters of the hemadsorption zones were measured. The dose response curve showed a linear relationship between the log of the serum dilution and the diameter of the zone, as has been shown by others (8). The slope of the curve was such that a 10-fold reduction of serum or antibody concentration corresponded to a 6.5-mm reduction of the diameter of the hemadsorption zone. In a few instances human sera showed nonspecific binding of immunoglobulins to the uninfected cell sheet (3 of 20 persons tested). These zones rarely exceeded the size of the paper disk and were not regularly reproducible. The reactions could be eliminated for both human and horse sera by absorption with uninfected cells. Hemadsorption zones smaller than 8 mm were regarded as nonspecific and considered negative.

(iii) Indirect IF test. The IF test was performed according to the standard method for rabies antibody determination (14). Human sera were tested in serial fourfold dilution series. Antigen substrate slides; (a) Impression smears of sectioned rabies-infected mouse brains were made on clean glass slides.

(b) Rabies-infected cells on cover slips in Leighton tubes: Monolayers were infected with 10⁴-5 TCID₅₀ of attenuated rabies virus by absorption for 1 h at 37°C. After 2 days, foci of infected cells showing intracytoplasmic inclusions of rabies antigen were seen. Slides were fixed in anhydrous acetone at ~20°C for 1 h and then stored at ~70°C until use.

The results of the indirect IF test were obtained by determining the highest serum dilution giving bright-green, distinct, typical intracytoplasmic inclusions in the cells. No attempt was made to evaluate immunofluorescence at the cell membrane. The same antibody titers were obtained for serum samples tested simultaneously on the two kinds of antigen slides.

Microscopy. A Zeiss standard universal microscope with incident light from an HBO 100-W high-pressure mercury lamp was used with exiter filter BG12 (350 to 450 nm) and barrier filters 41 and 50.

RESULTS

NT test. Table 1 shows the results of the NT tests. No antibodies were detectable in the preimmunization sera. All patients who received HDCV had detectable antibodies in the 14th-day serum sample. Individual serum antibody response to vaccination varied from 0.6 to 9.4 IU/ml (mean 3.8) and from 2.4 to 9.4 IU/ml (mean 8.0) in the 30th-day serum sample. There was an increase in titer in the second sample for all individuals except two in whom the titers were equal on days 14 and 30. Two patients vaccinated with DEV had no neutralizing antibodies in the lowest dilution (≥0.3 IU/ml) in the 14th-day serum sample. The positive sera in this group had low titers (0.6 to 2.4; mean 1.0). However, all patients had antibodies in the serum samples obtained on day 30 (0.3 to
4.7; mean 1.1). All 5 in the group of patients vaccinated with NTVs had titers on day 14 (0.3 to 7.1; mean 2.4) as well as on day 30 (2.4 to 18.9; mean 6.6). Thus, compared with DEV, titers were higher after HDCV on both days 14 and 30, whereas those after NTV were higher only on day 30.

**MH test.** Table 2 shows the proportion of positive sera by the MH test in the groups of patients immunized with the different vaccines. All patients vaccinated with HDCV had antibodies in the 14th- as well as the 30th-day serum samples, giving zones with a mean diameter of 13 and 17 mm, respectively. Sera from patients receiving DEV gave fewer and smaller reaction zones. In 14th- and 30th-day serum samples, four of eight and six of eight sera, respectively, gave positive reactions. In the group of patients vaccinated with NTV, the MH antibody response was also low: small zones were found in two and three of the patients on the two sampling days.

**Indirect IF antibody test.** All preimmunization sera were negative. Figure 1 shows the results with the different vaccines. Fourteen days after the beginning of immunization, more than half of the patients who received HDCV had titers of not more than 6.25, whereas none of the patients who received DEV and NTV had titers below 25. In the 30th-day serum sample, two patients in the HDCV group still had low titers (≤6.25). The highest titer (400) was found in the NTV group.

**Comparison of results obtained with the different test methods of sera from patients in the three vaccine groups.** The correlations between MH and NT titers for the three vaccine groups are shown in Fig. 2. In the HDCV group (Fig. 2A) there is good correlation between the results obtained with the two methods. All postimmunization sera contained antibodies demonstrable by both methods. Also, sera with relatively low NT titers (0.6 to 2.4 IU/ml) were positive by the MH test.

The MH and NT titers for the DEV and NTV groups are shown in Fig. 2B and C. In the two groups, 5 of 14 sera and 5 of 10 sera, with antibodies demonstrable by the NT test, were negative by the MH test. Because of the discrepancy between titers by the two tests, the immunoglobulins of four sera from the 14th-day serum sample in the NTV group were separated into sucrose gradients. Between 25 and 50% of the rabies-specific antibodies were shown, by the indirect IF test, to be of the immunoglobulin M (IgM) class. Only one low-titered serum (8-mm diameter) of the 15 MH positive sera in the two groups was negative by the NT test.

**DISCUSSION**

In this study, antibody responses to three types of rabies vaccine were used to evaluate tests for rabies antibodies. The NT test is the

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**Table 1. Results of NT**

<table>
<thead>
<tr>
<th>Day of serum sample</th>
<th>No. of sera with detectable antibodies/total no. of samples tested (IU/ml of serum)</th>
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<tbody>
<tr>
<td>HDCV*</td>
<td>DEV</td>
</tr>
<tr>
<td>0</td>
<td>0/8</td>
</tr>
<tr>
<td>14</td>
<td>8/8 (0.6-9.4);</td>
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<tr>
<td></td>
<td>mean 3.8</td>
</tr>
<tr>
<td>30</td>
<td>8/8 (2.4-9.4);</td>
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<td></td>
<td>mean 8.0</td>
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</tbody>
</table>

* Type of vaccine.

**Table 2. Results of the MH**

<table>
<thead>
<tr>
<th>Day of serum sample</th>
<th>No. of positives/no. of patients in each group*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDCV*</td>
<td>DEV</td>
</tr>
<tr>
<td>0</td>
<td>0/8</td>
</tr>
<tr>
<td>14</td>
<td>8/8 (8-16); 4/8 (8-12); 2/5 (11 and mean 13)</td>
</tr>
<tr>
<td>30</td>
<td>8/8 (15-20); 6/8 (8-17); 3/5 (8, 14 mean 17)</td>
</tr>
</tbody>
</table>

* Zone diameters of positive sera, ≥8 mm. Range of diameters (milliliters) for positive sera and mean diameter for positive results.

* Type of vaccine.
most logical test for estimating the protective capacity of an antibody response since it measures antibodies combining with the surface of the virus and has been shown to correlate with protection against virus challenge. In the past this has been the test used almost exclusively for estimating antibody responses to rabies vaccination. The use of the test to demonstrate the efficient immune reaction of humans to HDCV has been thoroughly documented (1, 3, 15). Antibodies have been detected as early as 7 days after the first injection (24). Bahmanyar (3), using the same immunization schedule (1-ml doses administered at 0, 3, 7, and 21 days) and the same source of vaccine as used in this study, invariably found NT antibodies by the 21st day. The neutralizing-antibody response after DEV has been found by some workers to be poor and opinion of the efficacy of this vaccine varies (20). In a report (5) where the neutralizing capacity was measured for a constant volume of test serum against varying amounts of infectious virus, antibody (in serum dilution 1:10) was found to be virtually absent in 5 of 13 patients immunized with 14 daily doses of vaccine. The NTV type of vaccine has been reported to elicit higher neutralization antibody titers than the DEV (18).

The MH test measures antibodies combining with virus-induced antigen on infected cells. In other test systems the surface of rabies-infected cells has likewise been shown to bind rabies virus antibodies (2, 9, 23). This antigen should be equivalent to the glycoprotein antigen present on the exterior of a budding virus. Providing antibodies of the different immune globulin classes are measured to the same extent, there should therefore be a correlation between antibody titers found in NT and MH tests.

The IF test mainly shows antibodies to the intracytoplasmic rabies nucleocapsid antigen and to a much lesser extent antibodies to the viral glycoprotein antigen present in infected cells. In fact, the antibody measured in this test may be induced by administration of isolated rabies nucleocapsid antigen. The discrepancy between indirect IF and NT results in a few individuals receiving duck embryo vaccine has been reported previously (11, 14). Up to 20% of postvaccination sera positive by the indirect IF test were negative by the NT test (14). These results might be attributable to the authors' use of two different antigens in the test and to the predominance of nucleocapsid antigen in the vaccine (5).

The extensively documented results reported by others for the NT test are confirmed in the present study. Thus, the HDCV group showed an antibody response by the NT test for all the 14th-day serum samples (0.6 to 9.4 IU/ml; mean 3.8). All individuals had an increase of antibodies in the 30th-day serum sample (2.4 to 9.4 IU/ml; mean 8.0) except two who had unchanged titers in both sera (Table 1). The neutralization antibody response of the DEV group was poorest (Table 1). In two individuals no antibodies were found in the lowest serum dilution in the 14th-day sample (<0.3 IU/ml), and the mean titer was low (1 IU/ml) for the six positive sera. In the 30th-day serum sample all individuals had positive but still rather low values (0.3 to 4.7 IU/ml; mean 1.1). The NTV group exhibited quite high levels of neutralizing antibodies (mean 2.4 and 6.6 IU/ml) for days 14 and 30, respectively. All sera in the HDCV group, 14th- and 30th-day, had antibodies measurable by the MH test (Table 2). For this group of vaccinees the NT and the MH tests had approximately the same degree of sensitivity (Fig. 2A). Furthermore, results from the two tests appear to correlate.

Only antigens exposed on the surface of the rabies virion induce antibodies associated with protection against the virus. Methods for determining antibodies after immunization with an inactivated vaccine should therefore use the glycoprotein of the rabies virus surface. This is the case with NT, MH, and HI tests. Of these, the NT and MH tests have been used here. The great sensitivity to nonspecific inhibition is a
serious drawback of the HI test and therefore this test has not been included in the study.

In the postexposure antibody response, a rapid appearance of antibodies of the IgG class is desired, as antibodies of the IgM class do not leave the vessels and thus have difficulty in reaching the locally introduced virus. Immunization with 14 daily doses of vaccine has been shown to prolong IgM antibody production (11, 16), probably due to the persistence of the antigen. In the present study, a total of 10 of 24 sera in the DEV and NTV groups with neutralizing antibodies were negative by the MH test. The NT test measures IgG as well as IgM and IgA antibodies, whereas the MH test as shown in model tests indicated only IgG antibodies. The IgM response in the immune reaction was verified by the high proportion (25 to 50%) of rabies IgM antibodies found by the indirect IF tests in the 14th-day serum sample in the NTV group. This has also been found earlier after DEV (11). The NT titers in Fig. 2B and C could thus partly be accounted for by IgM antibodies appearing during an immunization schedule with daily injections. These antibodies are not measured in the MH test and low NT titers for these two groups of vaccinees might therefore correspond to a negative MH test.

The present study shows the usefulness of the MH test for rabies antibody estimation after vaccination. Because it measures antibodies directed against virus surface antigens, the test correlated well with the NT test for the HDCV group. The MH test measured only antibodies of the IgG class, and it was sensitive and gave no false-positive results. This test also was feasible for quantitative antibody measurements, was much less tedious than the in vitro NT test, and gave a reliable result in 2 days.

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LITERATURE CITED