Sensitive Procedure for Detecting Residual Viable Virus in Inactivated Rabies Vaccine

J. R. MITCHELL, R. E. EVEREST, 1 AND G. R. ANDERSON

Bureau of Laboratories, Michigan Department of Public Health, Lansing, Michigan 48914

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A procedure for testing inactivated rabies vaccines of tissue culture origin for residual viable virus is reported in which the vaccine to be tested is passed in primary hamster kidney cell culture (PHK) before mouse inoculation. In preliminary experiments, titrations of rabies virus in which each dilution was passed in PHK before inoculating mice yielded titers 100 to 10,000 times higher than the titers obtained for the same virus by direct mouse inoculation. This rabies virus amplification procedure was evaluated by testing 18 lots of inactivated rabies vaccine of tissue culture origin. No viable virus was found in these vaccine lots when tested by direct intracerebral inoculation of mice. Eight of these 18 lots were found to contain viable virus, however, when tested by passage in PHK cell culture. The significance of low levels of viable virus in rabies vaccines is discussed. It is recommended that the amplification procedure described in this report be used in the safety testing of rabies vaccines of tissue culture origin and that it be evaluated for use in testing other rabies vaccines of low tissue content.

Several laboratories have devoted much effort, in the past few years, to the development of a rabies vaccine free from the encephalomyelitic factors present in the nerve tissue vaccines. Most of this effort has been directed toward a search for a new host for the virus or toward the development of better methods of purification and inactivation of the virus. In both approaches, the goal has been to obtain the protective antigen free from the factors which produce neurological complications. For all new methods of vaccine production, the question of completeness of inactivation of the virus must be raised. Any change in the method of producing the viral antigens may increase or decrease the effectiveness of the inactivating agent.

Although the standard method (3) of testing the nerve tissue vaccines for residual live rabies virus has apparently withstood the test of time, it cannot be assumed that the same method will be sufficiently sensitive to detect residual viable virus in the newer vaccines. Each safety test should be designed specifically for the vaccine to be tested and should be the most sensitive procedure that can reasonably be designed for that vaccine.

The following report describes a procedure for testing rabies vaccine of tissue culture origin for residual live virus in which the material to be tested is passed in primary hamster kidney culture before mouse inoculation. It is a simple procedure but it has detected rabies virus at dilutions more than 100 times higher than can be detected by direct intracerebral inoculation of weanling mice.

MATERIALS AND METHODS

Rabies vaccine. The hamster kidney rabies vaccines (HHRV) used in this study will be described in detail in subsequent publications. In brief, they are unconcentrated fluids from rabies virus-infected primary hamster kidney (PHK) cell cultures. Each pool of culture fluids was inactivated with 0.02% beta-propiolactone for 2 hr at 37 C.

Rabies virus. The rabies virus used is the CVS strain adapted to primary hamster kidney culture (1). It was obtained from Robert Kissling, Center for Disease Control, at the 112th PHK passage and was used in this study at the 116th PHK passage. Virus dilutions were made in tissue culture medium.

Mouse inoculation. Mouse inoculations were made by injecting 0.03 ml of material intracerebrally. If symptoms developed in a group of mice, the presence of rabies virus was confirmed in at least one animal from that group by the immunofluorescence rabies diagnostic technique.

The mice used in this study were either of the Michigan Department of Public Health strain of Swiss mice or the ICR strain obtained originally from Charles River, Inc. All of the mice used were grown in our production colony. Mice were used at 3 to 5 weeks of age.

Preparation and inoculation of primary hamster kidney cultures. Noninbred Syrian hamsters from our colony were used at 4 to 6 weeks of age. The kidneys

1 Present address: American Hoechst Corp., Animal Health Division, Kansas City, Mo. 64108.
were collected aseptically, minced into 2- to 3-mm fragments, and stirred in 0.2% trypsin at room temperature. The dispersed cells were pooled, centrifuged to remove the trypsin, and resuspended in growth medium to a convenient volume. The cells were planted in 32-oz prescription bottles at a concentration equivalent to one hamster kidney per bottle. The volume of medium in each bottle was brought to 50 ml, the cells were dispersed in the medium by shaking, and the bottles were incubated at 35 to 37 C.

The growth medium was Hanks balanced salt solution with 0.65% lactalbumin hydrolysate and 6% fetal calf serum. Maintenance medium was the same as the growth medium but with 2% fetal calf serum.

After 5 to 7 days of incubation, the growth medium was drained from the cell sheets and the inoculum was added. Incubation was continued for 90 min with gentle rocking of the bottles at 15- to 20-min intervals. The volume in each bottle was then brought to 50 ml with maintenance medium, and incubation at 35 C was continued for 14 days. Culture fluid was replaced 24 hr after inoculation and at 3- to 4-day intervals thereafter.

RESULTS

Relative sensitivity of mice and cell cultures in the detection of rabies virus. In preliminary experiments designed to measure the relative sensitivity of the new procedure, \( \log_{10} \) dilutions of rabies virus were made with sufficient volume at each dilution to permit the inoculation of one 32-oz (ca. 900 ml) bottle of PHK and five mice. Inoculum for each cell culture was 20 ml, and for each mouse 0.03 ml was given intracerebrally. Diluted virus was held at 4 C, and the time between mouse and PHK inoculation was kept to the minimum. This interval did not exceed 2 hr in any of the experiments.

Samples were removed from each inoculated PHK culture at 4, 8, 11, and 14 days postinoculation, and five mice were inoculated from each sample. Virus titers are expressed as the highest dilution which produced rabies death in at least one mouse during a 21-day observation period.

The possibility that the relatively large amount of inactivated virus in the vaccine might interfere with the adsorption of a small amount of live rabies virus to PHK cells was considered. Each experiment was performed in duplicate with tissue culture medium (EMEM) as diluent for one series of dilutions and inactivated rabies vaccine (HKRV) as diluent for a parallel series of dilutions.

In each of three such experiments (Table 1), at least 100 times more virus was detected by sequential PHK-mouse passage than by direct mouse inoculation. In one experiment, the difference was at least 10,000-fold. The virus was not diluted far enough to reach the end point. All samples were positive at the highest dilution, \( 10^{-8} \). There was no evidence that the presence of large amounts of inactivated rabies virus interfered significantly with the adsorption of live virus to PHK cells.

### Table 1. Comparison of rabies virus titer as determined by direct mouse inoculation and sequential tissue culture-mouse inoculation

<table>
<thead>
<tr>
<th>Virus no.</th>
<th>Diluent</th>
<th>Direct mouse inoculation</th>
<th>Sequential inoculation of tissue culture and mice*</th>
</tr>
</thead>
<tbody>
<tr>
<td>V160</td>
<td>EMEM&lt;sup&gt;a&lt;/sup&gt; HKRV&lt;sup&gt;d&lt;/sup&gt;</td>
<td>( 10^{-5} ) ( 10^{-6} )</td>
<td>( 10^{-8} ) ( 10^{-8} )</td>
</tr>
<tr>
<td>V157</td>
<td>EMEM    HKRV</td>
<td>( 10^{-5} ) ( 10^{-4} )</td>
<td>( 10^{-8} ) ( 10^{-8} )</td>
</tr>
<tr>
<td>V146</td>
<td>EMEM    HKRV</td>
<td>( 10^{-6} ) ( 10^{-5} )</td>
<td>( 10^{-8} ) ( 10^{-8} )</td>
</tr>
</tbody>
</table>

* Highest dilution which produced rabies in mice.

<sup>a</sup> Highest dilution tested was \( 10^{-4} \). Results were the same for samples taken at 4, 8, 11, and 14 days postinoculation.

<sup>d</sup> Eagle’s minimum essential medium.

Evaluation of the rabies virus amplification procedure. The use of tissue culture passage as a rabies virus amplification procedure (RVAP) was evaluated by studying two alternative procedures. In the first procedure, one 32-oz culture bottle of PHK was inoculated with 10 ml of the vaccine to be tested. In the second procedure, each of three to five culture bottles of PHK was inoculated with 20 ml of the vaccine to be tested. Each of the vaccines used in evaluating these two procedures was first tested for viable virus by the direct inoculation of 10 to 15 weanling mice.

With the first procedure, fourteen experimental lots of vaccine were examined for viable virus by the inoculation of one PHK culture with 10 ml of each vaccine. A sample of fluid was taken from each culture 8 and 14 days after inoculation, and five mice were inoculated from each sample. Although rabies virus could not be demonstrated in any of these vaccines by direct mouse inoculation, rabies virus was demonstrated in two lots after passage in PHK cultures.

The 12 lots of vaccine which were negative by the first procedure plus 4 additional lots were then tested by the second procedure (RVAP), which is now used as our routine test for residual viable rabies virus. For each lot of vaccine to be tested, three or five PHK cultures were each in-
Inactivated vaccines of probability intracerebral inoculation of the tested virus. However, this should be detected by the failure of the first procedure to detect the presence of viable virus in two lots of vaccine. Further study and experience under conditions of routine use will be required to define the best procedure for the detection of residual viable virus in rabies vaccines.

The use of suckling mice instead of weanling mice was not tested experimentally. We believe that the small increase in sensitivity which might be gained would not compensate for the added difficulty incurred, especially the nonspecific deaths in suckling mice which would have to be confirmed by some procedure such as immunofluorescence testing.

Further study of the optimal number of culture bottles and the optimal volume of inoculum per bottle is needed. The importance of the volume of inoculum and the number of culture bottles used is made evident by the failure of the first procedure to detect the presence of viable virus in two lots of vaccine. Further study and experience under conditions of routine use will be required to define the best procedure for the detection of residual viable virus in rabies vaccines.

The significance of a low concentration of viable rabies virus in a vaccine in terms of the risk to the human vaccinee cannot be studied directly. Many humans have, however, received vaccine containing viable virus. The Fermi-type vaccines (2), which intentionally contain up to 30,000 mouse LD50 of viable virus per 2-ml dose, have been used in humans for many years. Considering the failure of direct mouse inoculation to detect viable virus in the present study, it is not difficult to believe that a low concentration of viable virus has been present in many lots of the Semple-type vaccine which has been widely used in humans. The lack of evidence that these vaccines have caused rabies virus infection in humans gives little comfort, however, since they have been used in postexposure immunization, in which a vaccine-related rabies infection would be difficult to distinguish from a vaccine failure. The large antigenic mass of the nerve tissue vaccines may have protected against infection from residual viable virus. Such a high concentration of antigen is difficult to achieve in tissue culture.

The rabies vaccines now under development in our laboratory and elsewhere are intended to be used not only for postexposure prophylaxis but

**Table 2. Results of tests for residual viable virus in experimental rabies vaccine of tissue culture origin**

<table>
<thead>
<tr>
<th>Test method</th>
<th>No. of lots tested</th>
<th>No. of lots with residual viable virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct mouse inoculation</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>One culture bottle with 10 ml of inoculum</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Rabies virus amplification procedure</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>8</td>
</tr>
</tbody>
</table>

Occluded with 20 ml of vaccine. After a 90-min adsorption period, 30 ml of maintenance medium was added to each bottle and incubation was continued at 35°C for 14 days. Maintenance medium was replaced 24 hr after inoculation and every 3 or 4 days thereafter. Samples were removed from each bottle on days 8 and 14 after inoculation, and five mice were inoculated from each sample. A parallel titration of tissue culture-grown rabies virus in both mice and PHK cultures served as a control on the sensitivity of the procedure. Mice were inoculated with fluid from uninoculated PHK cultures as a control on the cell cultures. Six of these 16 vaccine lots contained residual viable rabies virus (Table 2).

Of the 12 lots which were negative for live virus when examined by the first procedure with 10 ml of inoculum in only one PHK culture, two additional lots were found positive by the second procedure in which each of five PHK cultures was inoculated with 20 ml of vaccine.

**DISCUSSION**

No live rabies virus could be demonstrated in 18 lots of experimental inactivated rabies vaccine by direct inoculation of mice. Viable virus was detected, however, in eight of these lots by passage of the vaccine in PHK culture.

The results of this study indicate that (i) the method of testing for viable virus by the intracerebral inoculation of mice is not of adequate sensitivity to detect the presence of a low concentration of residual viable rabies virus in inactivated vaccines of tissue culture origin, and (ii) the use of the RVAP procedure would greatly increase the probability of detecting any residual viable rabies virus present in such vaccines.

At least three factors contribute to the greater sensitivity of this procedure. (i) A larger volume of vaccine can be tested in cell cultures than can be tested in mice and this should increase the probability of detecting small amounts of infectious virus. (ii) The lack of defense mechanisms in cell cultures should make them more vulnerable to virus infection than the intact animal. (iii) The seed virus used to produce the vaccines tested was adapted to growth in PHK cultures; the virus might therefore have a greater affinity for the cell culture than for the mouse.

The first procedure studied in which only one PHK culture was inoculated with 10 ml of vaccine might be helpful in developmental work on a new vaccine, since it requires less material; it would not be sufficiently sensitive, however, for safety testing of inactivated vaccine intended for human use.

Further study of the optimal number of culture bottles and the optimal volume of inoculum per bottle is needed. The importance of the volume of inoculum and the number of culture bottles used is made evident by the failure of the first procedure to detect the presence of viable virus in two lots of vaccine. Further study and experience under conditions of routine use will be required to define the best procedure for the detection of residual viable virus in rabies vaccines.

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The rabies vaccines now under development in our laboratory and elsewhere are intended to be used not only for postexposure prophylaxis but...
also for preexposure immunization and in situations in which the probability of actual exposure to rabies virus is low. In such circumstances, the nerve tissue vaccines could be used only with great hesitancy, if at all. Before a rabies vaccine can be recommended for such wide use, every reasonable effort must be made to insure that it is free from viable rabies virus.

It is recommended that all inactivated rabies vaccines of tissue culture origin be tested for residual viable virus by the tissue culture passage amplification procedure before release for human use. This procedure should also be evaluated for use in testing other rabies vaccines which have a low tissue content.

ACKNOWLEDGMENT

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LITERATURE CITED