In vitro inactivation of the rabies virus by ascorbic acid

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Summary Objective: The current recommended inactivating agent for the rabies virus, beta propiolactone (BPL) is very expensive and potentially carcinogenic. There is a need to evaluate alternative chemicals, which will inactivate the virus without affecting its antigenicity. In this study the effect of ascorbic acid on the infectivity of the rabies virus has been investigated.

Method: Vero cell grown fixed rabies virus CVS strain was treated with 0.1 mg/ml, 0.5 mg/ml and 1 mg/ml final concentrations of ascorbic acid and 5 μg/ml of copper sulfate and kept at 4 °C along with untreated virus material. Each aliquot was titrated after various intervals for viral infectivity using both mice inoculation and titration in vero cells. The antigenicity of the virus material was determined by antibody induction in mice and modified NIH tests in parallel with virus material inactivated with a 1:4000 concentration of BPL.

Results: An optimal concentration of 0.5 mg/ml of ascorbic acid and 5 μg/ml of copper sulfate completely inactivated the virus after 72 hours. The inactivated virus retained good antigenicity and potency value, which was comparable with using BPL.

Conclusion: These findings suggest that ascorbic acid can be used as an inactivating agent for fixed rabies virus grown in cell culture particularly for the preparation of diagnostic reagents. Further studies are required to evaluate its effect on the cell associated virus, probable therapeutic potential and feasibility of replacing BPL in production of inactivated rabies vaccine.

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Introduction

The rabies virus is a single stranded negative sense RNA virus belonging to the genus Lyssavirus of the family Rhabdoviridae. The virus causes fatal encephalitis both in humans and animals, which is
still a major public health problem in developing countries in Asia, Africa and Latin America. Around 55,000 human deaths are still reported worldwide, mostly from the Indian subcontinent. Rabies is also re-emerging as an important infectious disease in North America where many human deaths have been associated with bat rabies. The production of vaccines, diagnosis and further research will therefore continue in this field.

Inactivation of this highly neurotropic virus is essential for the preparation of vaccines, diagnostic reagents and research purposes. Currently, the recommended inactivating agent for this virus is beta propiolactone (BPL), which is a very expensive chemical and potentially carcinogenic. Other chemicals like formaldehyde and phenol not only inactivate the virus but also adversely affect its antigenicity.

There is therefore a need to find alternative inactivating agents which are not expensive and are easily available. It was demonstrated by Turner et al. that ascorbic acid undergoing auto oxidation catalyzed by cupric ions inactivated vaccinia virus. Subsequently this was confirmed with other DNA and RNA viruses and it was also shown that the inactivated viruses retained good antigenicity. Recent studies have shown that this agent could also inactivate HIV and bacteriophages and interest has been renewed for using this chemical as an antiviral agent.

Keeping this in mind the effect of ascorbic acid on the rabies virus was investigated and the studies here indicate that this chemical along with cupric ions is capable of causing complete and irreversible inactivation of fixed rabies virus without adversely affecting the antigenicity.

Materials and methods

Virus material

The Vero cell adapted challenge virus strain (CVS 13, obtained from the Central Research Institute, Kasauli, India) was grown in Roux bottles on a monolayer of Vero cells (ATCC, CCL81, obtained from the National Center for Cell Science, Pune, India) by using standard procedures. The infectivity titer of the virus was determined by intracerebral inoculation of young mice using a standard procedure. Thus a stock virus material having a titer of 10^7 MLD50 (50% mouse lethal dose) per ml was prepared, aliquoted, and stored at −75 °C.

Chemicals and virus treatment

Ascorbic acid and copper sulfate were procured from Sigma Chemicals (USA). Stock solutions containing 0.1 M of copper sulfate and 0.5 M of ascorbic acid were prepared in sterile distilled water and sterilized by membrane filtration. The stock solutions were added to the virus suspension to obtain a final concentration of 5 µg/ml of copper sulfate and three increasing concentrations of ascorbate i.e. 0.1 mg/ml, 0.5 mg/ml and 1 mg/ml. The controls consisted of virus suspension to which equal volumes of sterile PBS (pH 7.2) was added. The test and the control virus material were kept at 4 °C. An aliquot was taken every 24 hours for seven days. There was no significant change in the pH of the virus material after addition of the chemicals.

Determination of infectivity titers

Each aliquot was titrated for viral infectivity using both mice inoculation and titration in Vero cells using the fluorescent focus method in 96 well tissue culture plates. Vero cells were grown as monolayers in the wells of the plate and 100 µl each of ten fold dilutions of virus material were added to each well, incubated for 1 hour at 37 °C after which fresh medium was added. The plates were further incubated for 72 hours in a CO2 incubator after which the cells were stained for immunofluorescence using rabies conjugate (obtained from the Central Research Institute, Kasauli, India). The plates were observed under a fluorescence microscope (Leitz) and the highest dilution of virus showing fluorescent foci in 50% of the microscopic field was noted. The exact titer was calculated by the Reed and Muench method.

For the inoculation, female four-week old Swiss albino mice were procured from the Central Animal Research Facility, NIMHANS. The animals were housed and looked after as per the regulations and permission to conduct the study was obtained from the Institutional Ethics Committee for experimentation on animals. A mouse LD50 titer of each aliquot was calculated by the Reed and Muench method after a 14-day observation of the inoculated mice. The fluorescent focus units (FFU/ml) titer was calculated after staining the Vero cells in the tissue culture plate with FITC rabies conjugate (obtained from the Central Research Institute, Kasauli) using standard procedures. The end point dilution showing 50% fluorescent foci in the well was noted and the titer was calculated by the Reed and Muench method. The infectivity titers of aliquots kept at −75 °C were also determined in parallel.
Studies to determine the antigenicity

Preliminary experiments had shown that treatment of the virus with 0.5 mg/ml of ascorbic acid for 72 hours completely inactivated it. Therefore, to determine the antigenicity of the inactivated virus we used virus material inactivated with 0.5 mg/ml of ascorbic acid and 5 μg/ml of copper sulfate. We determined the antigenicity of the virus material both by the induction of antibodies in experimental mice and by the mouse protection test. As a control we used the same viral harvest inactivated by BPL (Sigma Chemicals) at a concentration of 1:4000 kept for 72 hours at 4°C.

Groups of eight mice were inoculated intraperitoneally with two 0.5 ml doses of either ascorbic acid or BPL inactivated virus material at one-week intervals. They were then bled by the retro-orbital route one week later, after a light anesthesia with ether. The sera were separated and inactivated at 56°C for 30 minutes and kept frozen pending antibody titration by the rapid fluorescent focus inhibition test (RFFIT).

The RFFIT was performed as per the WHO recommended procedure13 but with some modification. Instead of Labtek chambers, 96 well tissue culture plates were used and the cell line used was BHK 21, CL 13 (obtained from the National Center for Cell Science, Pune). The challenge virus used was CVS adapted to BHK 21 cell line and having a titer of 10^7.8/ml when titrated in BHK 21 cells. For the test, 50 LD₅₀ of the challenge virus was used. The antibody titers were expressed as the reciprocal of the 50% end point dilution calculated by the method used by Reed and Muench. The titers were expressed in IU/ml so as to compare to a national standard of rabies vaccine (procured from WHO) having a potency of 16 IU per ampoule.

Results

Effects of ascorbic acid on virus infectivity

We tested the inactivating property of three concentrations of ascorbic acid keeping the concentration of copper sulfate constant. In the titers obtained by the mice inoculation and tissue culture method, there was only one log that was higher than the others.

Complete loss of infectivity of the virus treated by both methods with 0.5 mg/ml of ascorbic acid was evident at the end of the 72 hours (see Tables 1 and 2). With a 0.1 mg/ml concentration of ascorbic acid there was no appreciable reduction in the infectivity titer which remained at 10^6 at the end of seven days and did not differ significantly with the titer of virus control kept at 4°C.

Modified NIH test

The antigenicity of the inactivated virus material was also determined by the NIH test14 with some modifications. Groups of eight mice were inoculated intraperitoneally with 0.5 ml of 5 fold dilutions (1:5 to 1:3125) of both BPL and ascorbic inactivated virus material at one-week intervals. They were then challenged a week later with 50 LD₅₀ of CVS and observed for 14 days. The survival:death ratio in each dilution was noted and the 50% percent effective dose (ED₅₀) was calculated by the Reed and Muench method. The antigenicity in terms of potency was calculated so as to compare to a national reference standard previously calibrated against the fifth international standard of rabies vaccine (procured from WHO) having a potency of 16 IU per ampoule.

Table 1  The effect of ascorbic acid and Cu²⁺ on the infectivity titer of the vero cell grown CVS strain of the rabies virus as determined by intracerebral inoculation of mice.

<table>
<thead>
<tr>
<th>Virus suspension</th>
<th>Infectivity titer (Log MICLD₅₀/ml) at the end of day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Ascorbic acid treated</td>
<td></td>
</tr>
<tr>
<td>0.1 mg/ml</td>
<td>7.0</td>
</tr>
<tr>
<td>0.5 mg/ml</td>
<td>7.0</td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>7.0</td>
</tr>
<tr>
<td>Virus control</td>
<td></td>
</tr>
<tr>
<td>At 4°C</td>
<td>7.0</td>
</tr>
<tr>
<td>At -75°C</td>
<td>7.0</td>
</tr>
</tbody>
</table>

MICLD₅₀ = Mouse intracerebral 50% lethal dose.
Table 2 The effect of ascorbic acid and Cu²⁺ on the infectivity titer of the Vero cell grown CVS as determined by the fluorescent focus method using Vero cells.

<table>
<thead>
<tr>
<th>Virus suspension</th>
<th>Infectivity titer (FFU/ml) at the end of day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Ascorbic acid treated</td>
<td></td>
</tr>
<tr>
<td>0.1 mg/ml</td>
<td>6.2</td>
</tr>
<tr>
<td>0.5 mg/ml</td>
<td>6.0</td>
</tr>
<tr>
<td>1.0 mg/ml</td>
<td>6.0</td>
</tr>
<tr>
<td>Virus control</td>
<td></td>
</tr>
<tr>
<td>4 °C</td>
<td>6.2</td>
</tr>
<tr>
<td>−75 °C</td>
<td>6.5</td>
</tr>
</tbody>
</table>

FFU/ml = Fluorescent focus units/ml.

Inoculation and infection in cell culture dropped from $10^7$/ml to $10^3.5$/ml at the end of 24 hours and became zero at the end of 48 hours. However, with this concentration the pH of the medium became acidic (from pH 7.4 to pH 6.5). With a concentration of 0.5 mg/ml of ascorbic acid the infectivity titer fell slowly compared to the higher concentration but complete inactivation was noticed at the end of 72 hours. There was no significant change in the pH. Therefore, for all future experiments, an ascorbic acid concentration of 0.5 mg/ml was fixed.

Induction of neutralizing antibodies in mice

Both ascorbic acid and BPL inactivated virus material induced comparable levels of neutralizing antibodies as evidenced by RFFIT (Table 3). Groups of eight mice were inoculated with the inactivated virus material and a very good neutralizing antibody response was obtained in all the mice inoculated. The geometric mean titer (GMT) of the ascorbic acid inactivated virus was 3.5 IU/ml and that of BPL inactivated virus was 3.1 IU/ml and so they did not differ significantly.

Potency

To further confirm the antigenicity of the inactivated virus material, a modified NIH test was performed, which involved the immunization of mice with the inactivated virus followed by a lethal challenge with the live virus. It is evident from the results of the modified NIH test that the ED₅₀ of both ascorbic acid and BPL inactivated virus materials did not differ significantly and the potency values obtained were comparable to a reference vaccine (Table 4).

Discussion

Earlier experiments by Turner et al.⁵ and more recent studies by Rawal et al.⁶,⁷ have indicated that ascorbic acid can be used as an inactivating agent for both RNA and DNA viruses. To our knowledge this is the first controlled study to investigate the inactivating property of these chemicals on the rabies virus.

Table 3 Neutralizing antibody titers (IU/ml) in mice inoculated with BPL and the ascorbic acid inactivated rabies virus.

<table>
<thead>
<tr>
<th>Virus type</th>
<th>RFFIT titers (IU/ml) in mice numbered GMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>BPL inactivated</td>
<td>2.4</td>
</tr>
<tr>
<td>Ascorbic acid inactivated</td>
<td>3.4</td>
</tr>
</tbody>
</table>

RFFIT = Rapid fluorescent focus inhibition test, GMT = Geometric mean titer.

Table 4 The ED₅₀ and antigenic potency of BPL and ascorbic acid inactivated virus material as determined by a modified NIH test.

<table>
<thead>
<tr>
<th>Virus material</th>
<th>ED₅₀</th>
<th>Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid inactivated</td>
<td>1:625</td>
<td>3.5 IU/ml</td>
</tr>
<tr>
<td>BPL inactivated</td>
<td>1:700</td>
<td>3.8 IU/ml</td>
</tr>
</tbody>
</table>

ED₅₀ = 50% effective dose.
The minimum effective concentration of ascorbic acid was found to be 0.5 mg/ml and at this concentration there was no significant fall in pH. Increasing the concentration to 1 mg/ml resulted in significant fall in pH and this caused a sloughing of Vero cells during titration. A concentration of 0.5 mg/ml was therefore used in all following experiments to determine the antigenicity of the inactivated virus. At this concentration there was complete and irreversible inactivation of the virus at the end of 72 hours.

The virus remained non-infectious at the end of seven days. One of the criteria for selecting an inactivating agent for any virus (unless for therapeutic purposes) is that it should not adversely affect the antigenicity of the virus so that the inactivated virus could be used as a vaccine or for any other research purpose.

Additionally, the rabies virus is highly neurotropic and can cause fatal encephalitis and so necessary precautions should be taken while handling the virus and unless indicated the live virus should not be handled in routine laboratories. As the use of BPL is very expensive, cheaper chemicals need to be evaluated for their inactivating properties.

Results of the present study indicate that ascorbic acid, which is not only inexpensive but also easily available, is found to be suitable for this purpose.

The mechanism by which ascorbic acid inactivates viruses is not very clear. A recent study by Murata et al.8 has shown that the presence of oxygen is essential and ascorbic acid undergoing auto-oxidation results in the formation of OH groups that could bring about the inactivation of the cell free viruses. It remains to be established whether ascorbic acid could inactivate cell associated viruses. This will be of great interest particularly if ascorbic acid is considered as a potential therapeutic agent. Indeed, in a study by Gode et al.15 the lives of two rabies patients were prolonged with the administration of high doses of ascorbic acid. Another possibility is its potential for local wound infiltration in order to inactivate the virus deposited after animal bites, especially in view of the fact that there may be global shortage of rabies immunoglobulins in the near future.16

Conclusion

It has been demonstrated that ascorbic acid has a potential for being used as an inactivating agent for the rabies virus without adversely affecting its antigenicity. Further studies are required to determine whether it is feasible to use this chemical for the production of inactivated rabies vaccines and whether it has therapeutic potential for treating rabies patients.

Conflict of Interest: No conflicting interest declared.

References

3. MMWR. Human rabies – Texas and New Jersey, 1997;47:1–5