INFLUENCE OF ENCEPHALITOZOOON CUNICULI EXPERIMENTAL INFECTION ON ANTIRABIES IMMUNITY IN A MOUSE MODEL

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Abstract

The influence of an immunosuppressive factor – infection with Encephalitozoon cuniculi – on the immunogenic, antigenic, and protective activity of rabies vaccines as well as on the nonspecific resistance against rabies infection in model experiments on mice was investigated. The results indicated a meaningful immunosuppressive influence of the E. cuniculi infection. The immunogenic activity of rabies vaccines was reduced to 67% in suppressed (infected with E. cuniculi) animals in comparison with healthy (intact) individuals. Similarly, the protective activity was also decreased. The quantification of rabies antibodies on days 14 and 21 after vaccination showed significantly lower titres in the E. cuniculi infected group of animals in comparison with the intact group; on day 28 the decrease was not significant. The effect of immunosuppression after experimental E. cuniculi infection was manifested also by the low value of the half-lethal-dose (MSCLD50) – 10–1.99 in intact animals and 10–2.71 in the suppressed ones.

Key words: mice, rabies, immunity, experimental infection, Encephalitozoon cuniculi, immunosuppression.

Safety and efficacy are the basic criteria in evaluating vaccine quality (including rabies vaccines). The antigenic and immunogenic activity are the criteria of efficacy determination. For rabies vaccines, the latter one is usually carried out in in vivo model experiments on laboratory animals. In the process of the development of new vaccines the efficacy determination is also carried out in target animals. Immunogenic activity evaluation is based on the challenge of vaccinated animals. Antigenic activity is established by quantification of the antibody titre. Evaluation of the immunogenic activity of rabies vaccines is internationally regulated by recommendations and standards – methodical procedures of the WHO (19), OIE (10) and European Pharmacopoeia (2). The methodical procedures have been standardized on an international level (5, 7, 13, 20), however, they are also characterized by several insufficiencies (17). We tried to eliminate these insufficiencies by proposals of altered methods of the injection as well as oral rabies vaccine efficacy testing (16, 17). The use of international standards enables to express the results in international or equivalent units (IU/cm² or in EU/cm²). The use of conventional (not specific pathogen free – SPF) laboratory animals in challenge experiments may influence the experiment results because of different immunosuppressive factors.

In evaluation of the immunogenic and antigenic activity of rabies vaccines in model experiments on laboratory mice, the infection with a parasite Encephalitozoon cuniculi can often be a hidden cause of the results deformation because of its immunosuppressive effects. E. cuniculi infection, among others, is a cause of decreased phagocytic activity (6), decreased numbers of peripheral lymphocytes and neutrophils, and increased numbers of monocytes (9).

The aim of our experiment was to determine how E. cuniculi experimental infection influenced the immunocompetence of vaccinated animals and immunogenic and antigenic values of rabies vaccines tested on laboratory mice. Our intention was to experimentally prove the necessity of laboratory animals examination prior to using them in immunological experiments and the obligation of SPF breeding declaration.

Material and Methods

Animals. The experiments were carried out on 279 SPF laboratory BALB mice (adult males) from the
Top Dovo breeding (Dobrá Voda pri Trnave, Slovak Republic) weighing 20 and 18 g. The research was conducted according to the principles presented in the „Guide for Care and Use of Laboratory Animals“, published by the Government of Slovak Republic (4).

**Vaccines.** Two parenteral rabies vaccines were used:
- standard international inactivated rabies vaccine (SI, Copenhagen, Denmark);
- commercial rabies inactivated Lyscelin vaccine, charge Nr. 625207 (Bioveta, Ivanovice na Hané, Czech Republic).

**Culture of E. cuniculi.** *E. cuniculi* was cultured in a VERO E-6 cell culture according to Halánová et al. (6).

**Immunosuppressing of mice.** The mice were infected intraperitoneally with *E. cuniculi* at a dose of 0.2 cm³/1.10⁷ particles. Serological control was carried out simultaneously with the challenge. On the basis of preliminary titration, the serological control carried out in 5 infected mice gave positive results. Rabies virus (CVS strain) titration was carried out in 5 suppressed mice and 21 intact mice. The serological control carried out in 5 suppressed mice gave positive results. Twenty-one suppressed and 21 intact mice were vaccinated subcutaneously into the upper chop with 0.2 cm³ of Lyscelin vaccine at a dose of 1 ED₅₀.

Challenge of 7 immunized mice was carried out on days 14, 21 and 28 after vaccination, respectively, applying 0.1 ml of the CVS virus strain at a dose of 2 MSCLD₅₀ (on the basis of preliminary titration) into the upper chop. The protective activity of the vaccine was expressed in percentage of survival rate after challenge; survival was observed for 1 month after challenge.

**Determination of dynamics of rabies vaccine antigenic activity.** The determination of the protective activity of *Lytic* vaccine was carried out on 26 suppressed and 21 intact mice. The serological control carried out in 5 suppressed mice gave positive results. Twenty-one suppressed and 21 intact mice were vaccinated subcutaneously into the upper chop with 0.2 ml of Lyscelin vaccine at a dose of 2 ED₅₀ (a dose providing 100% protection according to the preliminary experiments). On days 14, 21 and 28, blood samples were obtained from the caudal vein of the animals. Detection and quantification of rabies antibodies were carried out simultaneously by the ELISA (expressed in equivalent units EU/cm³) and RFFIT (in IU/cm³).

Statistical evaluation of rabies antibody titration was carried out by means of the Student’s t-test.

**Influence of immunosuppression on non-specific resistance towards rabies infection.** In the experiment 55 mice, each of 18 g body weight, were used. Thirty animals were infected with *E. cuniculi*. The serological control carried out in 5 infected mice gave positive results. Rabies virus (CVS strain) titration was carried out in 25 suppressed and 25 intact mice. Serial dilutions 1:20–1:320 were used. The individual dilutions were applied subcutaneously into the upper chop to 5 animals each at a volume of 0.1 ml. The result are expressed as virus titre in MSCLD₅₀/0.1 cm³.

## Results

When determining the immunogenic activity of the tested rabies vaccines by the LPL method, the antigenic value of vaccine determined by the using of suppressed mice (after experimental *E. cuniculi* infection) achieved only 67% of the value by using intact mice in the test (Table 1). The results confirmed the immunosuppressive effect of *E. cuniculi* infection and modulatory influence of subclinical *E. cuniculi* infection of mice on the value of rabies vaccine immunogenic activity.

The immunosuppressive effect of experimental *E. cuniculi* infection was proven also by the dynamics of protective activity of rabbits Lyscelin vaccine. In
suppressed mice survival rates of 42.85% and 71.43% were observed on days 14 and 21 after challenge, respectively. The rates were markedly lower than those in intact mice, especially on day 14. The survival rate on day 28 was identical in both groups of animals – 71.43%. The results are summarized in Table 2.

The dynamics of rabies Lyscelin vaccine antigenic activity was evaluated by rabies antibody titre determination. The results are summarized in Table 3. In the group of suppressed mice, the mean titres of rabies antibodies determined by ELISA were significantly lower in comparison with the intact group on days 14 (P<0.005) and 21 (P<0.025), on day 28 there was not significant decrease because of the great intra-group dispersion. Similar results were obtained by RFFIT, the statistical significance was slightly higher on day 21 (P< 0.01) comparing with the ELISA.

Observations of the immunosuppressive effect of *E. cuniculi* infection on non-specific resistance against rabies infection revealed an explicit immunosuppressive effect when the results of rabies virus fixe strain CVS titration were compared between the suppressed and the intact mice. In intact mice, mortality was lower (MSCLD<sub>50</sub>/0.1 ml = 10<sup>-1.99</sup>) than in the suppressed animals (MSCLD<sub>50</sub>/0.1 ml = 10<sup>-2.71</sup>).

### Table 1
Results of rabies vaccine immunogenic activity determination by LPL method in experiments on intact and suppressed (experimentally *E. cuniculi* infected) mice

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>ED&lt;sub&gt;50&lt;/sub&gt; (µl)</th>
<th>AV (IU/cm&lt;sup&gt;3&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>0.000186208</td>
<td>16.00</td>
</tr>
<tr>
<td>Lyscelin – intact mice</td>
<td>0.0014187</td>
<td>2.10</td>
</tr>
<tr>
<td>Lyscelin – <em>E. cuniculi</em> infected mice</td>
<td>0.00218</td>
<td>1.40</td>
</tr>
</tbody>
</table>

ED<sub>50</sub> – effective dose 50%

AV – antigenic value

IU/ml – international units in 1 ml

### Table 2
The effect of immunosuppression (*E. cuniculi* infection) on the dynamics of protective activity of rabies Lyscelin vaccine

<table>
<thead>
<tr>
<th>Challenge day after vaccination</th>
<th>Survival after challenge (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Suppressed mice</td>
</tr>
<tr>
<td>14</td>
<td>42.85</td>
</tr>
<tr>
<td>21</td>
<td>71.43</td>
</tr>
<tr>
<td>28</td>
<td>71.43</td>
</tr>
</tbody>
</table>

### Table 3
Rabies antibody titres in suppressed and intact mice at different times after vaccination with rabies Lyscelin vaccine

<table>
<thead>
<tr>
<th>Group</th>
<th>Days after vaccination</th>
<th>ELISA (EU/ml)</th>
<th>RFFIT [IU/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressed</td>
<td>14</td>
<td>0.111 ± 0.039 ****</td>
<td>0.098 ± 0.035 ****</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0.271 ± 0.159 **</td>
<td>0.221 ± 0.121 ***</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.632 ± 0.238</td>
<td>0.562 ± 0.219</td>
</tr>
<tr>
<td>Intact</td>
<td>14</td>
<td>0.473 ± 0.218</td>
<td>0.412 ± 0.189</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0.630 ± 0.295</td>
<td>0.620 ± 0.286</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.858 ± 0.335</td>
<td>0.612 ± 0.326</td>
</tr>
</tbody>
</table>

** P < 0.025; *** P < 0.01; **** P < 0.005

EU/ml – equivalent units in 1 ml

IU/ml – international units in 1 ml
Discussion

Vaccine quality is mainly determined on the basis of its safety and efficacy, i.e. immunogenic and antigenic activity testing. For the evaluation of the immunogenic activity several international standardized methods on laboratory animal are used (5, 7, 13, 20). However, the latter have several shortcomings; they insufficiently imitate the conditions of the target animals vaccination. In addition, the way of challenge does not imitate the way of natural rabies infection. On the basis of earlier experiments as well as the comparison of several methods, we consider the LPL method developed in our laboratory (16) to be the most suitable one.

However, the result reliability is depending on several factors. In addition to the evaluation method choice, many different immunomodulating factors can affect the results of the vaccine immunogenic and antigenic activity quantification and thus de facto decide about their further use. Encephalitozoon cuniculi infection is one of the immunosuppressive factors frequently occurring in laboratory animal breedings (6). In E. cuniculi infected immunocompetent animals and humans clinical signs are rarely recorded. A chronic asymptomatic course of E. cuniculi infection also distorts the results of immunological experiments (18). Our experiments confirmed this assumption.

In experiments aimed at the rabies vaccine immunogenic activity determination we observed significant differences when comparing suppressed (E. cuniculi infected) and intact mice in the tests. Immunosuppression caused the relative decrease in the antigenic value of the tested vaccines in E. cuniculi infected mice from 2.1 IU/ml to 1.4 IU/ml. Halámová et al. (6) observed a decrease in the phagocytic activity after intraperitoneal administration of E. cuniculi to mice. Phagocytic activity decreased with the gradual increase in E. cuniculi antibody levels. Decreased phagocytic activity (6) together with decreased peripheral lymphocyte and neutrophil counts as parameters of the E. cuniculi infection immunosuppressive effect (9) probably reduces the immunocompetence after rabies vaccination.

Our further experiments also confirmed the facts given above. The determination of dynamics of vaccine antigenic activity showed significantly decreased levels of rabies antibodies of experimentally infected laboratory animals when compared to control ones. Survival rate of suppressed mice on days 14, 21, and 28 after challenge was significantly decreased in comparison with the control intact group. The immunosuppression caused also a decrease in animal non-specific resistance. When suppressed mice had been infected with rabies CVS virus, mortality increased in comparison with the controls.

The growth of the parasite E. cuniculi and the persistent production of antibodies are in balance and causes an asymptomatic course of the disease (6). Clinical manifestation of E. cuniculi infection is conditioned by the immune system depression by another pathogen or immunosuppressive factor (8). Our research was oriented on the partial immunosuppressive effect of E. cuniculi which was manifesting after immune system load.

In order to ensure the objectivity of immunological experiments on laboratory animals, prior to using the animals in experiment, generally at least a serological screening of E. cuniculi antibodies in breedings is inevitable. An extended certificate of experimental laboratory animals in SPF breedings with respect to immunosuppressive effects of some infections could be a solution.

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References


