For reasons of rationalization and objectivization of the in vivo rabies vaccine potency testing the authors compared different, routinely used routes of vaccine administration (subcutaneous, intramuscular and intradermal), as well as different ways of challenge that correspond to the natural ways of exposure (subcutaneous, intramuscular) of target animal species with the referent NIH method (intraperitoneal immunization, intracerebral challenge). Immunogenic and antigenic activity of rabies vaccines and the respective correlations were investigated. On the basis of low correlation rate of the immunogenic and antigenic activity values of rabies vaccines tested, the authors consider the NIH method non-objective. A high correlation rate between immunogenic and antigenic activity of tested vaccines was recorded by using the LPL method. The authors used an alternative in vitro method – simplified ELISA test for the quantification of rabies antigen content in the tested vaccines. The method can be used only for non-adjuvant vaccines. According to the authors, the antigen content corresponding to 1.0 EU/cm$^3$ is considered the lower limit for satisfactory efficacy of the vaccine.

Key words: antigen content, antigenic value, immunogenic activity, vaccine efficacy

INTRODUCTION

The basic requirements on vaccines applied in human and veterinary immunoprophylaxis is their safety and protection of human and animal health, especially, their ability to induce an adequate immune reaction capable of ensuring protection against infection. Rabies vaccines must also comply with these requirements and their effectiveness is one of the basic factors in the control of rabies, this being one of the most dangerous zoonoses. Testing of rabies vaccines is oriented toward the evaluation of their harmlessness with particular stress on determination of their effectiveness. The in vivo tests used for the determination of the efficacy of rabies vaccines are based on the evaluation of their immunogenic activity by quantitative methods and as well on the determination of the virus or viral glycoprotein content in the tested vaccines (WHO, 1996).
Despite the availability of standard testing procedures, the objectivity of these tests raises some doubts among researchers and remains a currently unsolved issue (Cussler et al., 1998). The current standard procedures of evaluation of rabies vaccine efficacy do not correspond to natural conditions of immunization and challenge of target animals (Wunderli et al., 2003a; 2003b).

In order to simplify the evaluation procedures, reduce costs of testing, but still considering the ethical aspects, efforts have been made to use in vitro methods for the evaluation of the efficacy of rabies vaccines (Bruckner et al., 1989; Wilbur, 1993; Gamoh et al., 1996, 2003; Cussler et al., 1998). Rooijakkers et al., (1996b) used the ELISA system for antigenicity testing of rabies vaccines, however, they found it more suitable for human vaccines than for veterinary ones.

Our study compared different ways of evaluation of the efficacy of different injection routes of rabies vaccines. To adjust the test to natural conditions we used alternative ways of immunization and challenge and compared them with the NIH reference method (WHO, 1996). The second objective of the paper was to compare the rabies vaccine antigen content, obtained by simplified ELISA test with the antigenic values obtained by an in vivo experiment.

MATERIAL AND METHODS

Experimental animals: random-bred white mice (ICR strain), body weight 12 g were used. The research was conducted according to the principles presented in the "Guide for Care and Use of Laboratory Animals", published by the Government of the Slovak Republic, No. 289/2003.

A) In vivo methods of evaluation of rabies vaccines efficacy – effects of vaccination mode and challenge route on immunogenic and antigenic activity of injected rabies vaccines

1) Rabies vaccines used in the experiment:
   a) Commercial veterinary inactivated vaccine Rabisin (Merial, France);
   b) Commercial veterinary inactivated vaccine Rabicell (Mevak a.s. Nitra, Slovak Republic);
   c) International referent vaccine (16 IU/cm³); (Statens Seruminstitut, Copenhagen, Denmark).

2) Determination of the immunogenic activity of rabies vaccines:
   - The immunogenic activity of rabies vaccines is established on the basis of determination of 50% effective dose (ED₅₀ vaccine dose that protects 50% of the immunized animals). In this part of the study we determined the immunogenic activity of rabies vaccines by four different methods. Mice weighting 12 g were immunized using the vaccine volume adequate to the immunization route (Table 1). The same dilution was used for each method (1:25 – 1:15625, dilution factor = 5). Challenging was performed by different means with virus suspension volumes corresponding to the respective infection route (Table 1). The challenge CVS virus...
was diluted for each method on the basis of preliminary titration to ensure the following infectious doses: a) method NIH – 30* MICLD_{50} (quantification by control titration); b) method LPL – 2** MSCLD_{50}; methods i.m./i.m. and i.d./i.m. – 2*** MIMLD_{50}. Preliminary titrations were performed on mice of same weight categories as those reached by the experimental animals at the time of challenge.

Table 1. Schedule of immunogenic activity evaluation of tested rabies vaccines (Rabisin, Rabicell)

<table>
<thead>
<tr>
<th>Method</th>
<th>n</th>
<th>Immunization route</th>
<th>Inoculum volume</th>
<th>Challenge route</th>
<th>Inoculum volume</th>
<th>Day of challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH</td>
<td>10</td>
<td>i.p. (0. and 7. day)</td>
<td>0.5 cm^3 2-times</td>
<td>i.c.</td>
<td>0.03 cm^3</td>
<td>day 14 after 1st immunization</td>
</tr>
<tr>
<td>LPL</td>
<td>5</td>
<td>s.c.</td>
<td>0.2 cm^3</td>
<td>s.c.</td>
<td>0.1 cm^3</td>
<td>day 21 after immunization</td>
</tr>
<tr>
<td>i.m./i.m.</td>
<td>5</td>
<td>i.m.</td>
<td>0.2 cm^3</td>
<td>i.m.</td>
<td>0.1 cm^3</td>
<td>day 21 after immunization</td>
</tr>
<tr>
<td>i.d./i.m.</td>
<td>5</td>
<td>i.d.</td>
<td>0.02 cm^3</td>
<td>i.m.</td>
<td>0.1 cm^3</td>
<td>day 21 after immunization</td>
</tr>
</tbody>
</table>

n - number of mice in each group
i.p. - intraperitoneal
i.c. - intracerebral
s.c. - subcutaneous into the upper shoulder
i.m. - intramuscular into the thigh
i.d. - intradermal into the nose tip
LPL - Rabies Research Laboratory (University of Veterinary Medicine, Košice, Slovak Republic)

– On the basis of survival rate of experimental animals the effective ED_{50} doses were calculated according to Reed and Muench (1938). By comparing the ED_{50} of the tested vaccines and the referent one, the immunogenic activity can be expressed in international units (IU/cm^3) (antigenic value – AV; the antigen amount in vaccine volume unit).

– The schedule of the experiments for the evaluation of immunogenic activity of the tested rabies vaccines (Rabisin, Rabicell) is presented in Table 1.

3) Determination of antigenic activity of rabies vaccines:

– The antigenic activity of rabies vaccines was determined on the basis of their ability to induce the production of specific antibodies at the respective immunization route. Mice weighting 12 g were immunized by the same route as those used in immunogenic activity determination experiments, i.e. about 10 animals from each group were immunized by i.c., s.c., i.m. or i.d. route. The level of rabies antibodies was determined in time intervals when the animals had to be challenged – i.e on day 14 after the first immunization by the NIH method and on day 21 by other methods (Table 2).

* MICLD_{50} – mouse intracerebral lethal dose 50%;
** MSCLD_{50} – mouse subcutaneous lethal dose 50%;
***MIMLD_{50} – mouse intramuscular lethal dose 50%
The schedule of experiments for antigenic activity evaluation of the tested rabies vaccines (Rabisin, Rabicell) is presented in Table 2.

Table 2. Schedule of antigenic activity evaluation of tested rabies vaccines (Rabisin, Rabicell)

<table>
<thead>
<tr>
<th>Immunization route</th>
<th>Inoculum volume</th>
<th>Dose</th>
<th>Day of blood sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.p. (2-times)</td>
<td>0.5 cm³</td>
<td>100 ED50</td>
<td>day 14 after 1st immunization</td>
</tr>
<tr>
<td>s.c.</td>
<td>0.2 cm³</td>
<td>100 ED50</td>
<td>day 21 after immunization</td>
</tr>
<tr>
<td>i.m.</td>
<td>0.2 cm³</td>
<td>100 ED50</td>
<td>day 21 after immunization</td>
</tr>
<tr>
<td>i.d.</td>
<td>0.02 cm³</td>
<td>100 ED50</td>
<td>day 21 after immunization</td>
</tr>
</tbody>
</table>

Explanation in Tab.1.
n – number of mice in each group = 10
ED50 – 50% effective dose

Serological examination – the rabies antibodies titres in mice sera were detected by ELISA method using the ELISA kit developed in our laboratory (Beníšek et al., 1989; Süliová et al., 1994). Statistical evaluation of results of rabies antibody titres was carried out by Student t-test.

B) In vitro method of evaluation of rabies vaccine efficacy

- Rabies vaccines used in the experiment:
  a) Live tissue culture infectious medium (live rabies vaccine) from strain Vnukovo-32/107;
  b) Inactivated tissue culture infectious medium (inactivated rabies vaccine) from strain Vnukovo-32/107;
  c) Commercial veterinary inactivated vaccine Rabicell – vaccination strain: Vnukovo-32 (Mevak a.s. Nitra, Slovak Republic);
  d) International reference vaccine (16 IU/cm³); (Statens Seruminstitut, Copenhagen, Denmark).

- Detection of immunogenic activity by the in vivo LPL method:
The immunogenic activities of the vaccines were determined by the LPL method (above described, Tab.1) and expressed as ED50 of vaccine. The antigenic value is given in international units IU/cm³.

- Quantification of antigen content by in vitro method:
In addition to antigenic value of rabies vaccines we determined also the antigen content by in vitro ELISA test. Quantification of the antigen content of the tested rabies vaccines was carried out by sandwich ELISA method for rabies antigen determination (Van der Marel and Van Wezel, 1981), modified by Rabies Research Laboratory of the University of Veterinary Medicine, Košice (Slovak
The microtitration plates were sensibilized by rabbit rabies immunoglobulins and overlayed with rabies antigen (vaccine) at $2^n$ serial dilutions. Human rabies immunoglobulins served as the second antibody. Anti-human immunoglobulins from swine marked with enzyme peroxidase (SwaHu/IgG-Px, SEVAC Praha, Czech Republic) were used as conjugate. The calibration curve was constructed by means of positive and negative (tissue culture medium without antigen) antigen at serial dilutions $2^n$. The antigen content in the samples was calculated from the calibration curve.

**RESULTS**

The immunogenic activity of vaccines Rabisin and Rabicell, determined by the LPL method, differed somewhat from that determined by other methods. It indicated higher efficacy of Rabicell while other methods showed higher immunogenic activity of Rabisin (Table 3).

Table 3. Results of immunogenic activity of rabies vaccines tested by different immunization routes

<table>
<thead>
<tr>
<th>Immunization method</th>
<th>n</th>
<th>Rabisin ED$_{50}$ [mm$^3$]</th>
<th>Rabisin IA [IU/cm$^3$]</th>
<th>Rabicell ED$_{50}$ [mm$^3$]</th>
<th>Rabicell IA [IU/cm$^3$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH</td>
<td>10</td>
<td>$1.501 \times 10^{-3}$</td>
<td>2.007</td>
<td>$1.777 \times 10^{-3}$</td>
<td>1.838</td>
</tr>
<tr>
<td>LPL</td>
<td>5</td>
<td>$2.786 \times 10^{-3}$</td>
<td>1.576</td>
<td>$2.551 \times 10^{-3}$</td>
<td>1.865</td>
</tr>
<tr>
<td>i.m. / i.m.</td>
<td>5</td>
<td>$3.965 \times 10^{-3}$</td>
<td>1.238</td>
<td>$4.087 \times 10^{-3}$</td>
<td>1.201</td>
</tr>
<tr>
<td>i.d. / i.m.</td>
<td>5</td>
<td>$1.880 \times 10^{-2}$</td>
<td>0.826</td>
<td>$2.051 \times 10^{-2}$</td>
<td>0.757</td>
</tr>
</tbody>
</table>

Explanation in Tab.1.

n – number of mice

ED$_{50}$ – 50 % effective dose

IA – immunogenic activity

IU – international units

The antigenic activity of both vaccines was adequate only when they were administered by i.p. and s.c. routes – the rabies antibodies levels reached the standard value of 1.0 EU/cm$^3$ (determined by ELISA method; WHO, 1996) at the time of challenge only when administered by above mentioned immunization routes. The differences between antirabies antibody levels with different immunization methods were insignificant because of the considerable intergroup dispersion of the results (Table 4).

Correlations between the immunogenic and antigenic activities of the tested vaccines showed a low correlation between the immunogenic activity of Rabisin and Rabicell (Table 3), determined by the NIH method. Antigenic activity (Table 4) of the vaccines was recorded after corresponding immunization (intraperitoneal). The highest correlation rate between immunogenic and antigenic activities of the
tested vaccines was found with the LPL method (subcutaneous route of immunization and challenge of mice).

Table 4. Results of antigenic activity (antibody titres in EU/cm³) of rabies vaccines tested by different immunization routes

<table>
<thead>
<tr>
<th>Immunization method</th>
<th>Number of doses</th>
<th>Rabisin</th>
<th>Rabicell</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.p.</td>
<td>2</td>
<td>1.01 ± 0.96</td>
<td>1.05 ± 1.10</td>
</tr>
<tr>
<td>s.c.</td>
<td>1</td>
<td>1.65 ± 1.23</td>
<td>2.50 ± 2.00</td>
</tr>
<tr>
<td>i.m.</td>
<td>1</td>
<td>0.52 ± 0.33</td>
<td>0.71 ± 0.68</td>
</tr>
<tr>
<td>i.d.</td>
<td>1</td>
<td>0.35 ± 0.29</td>
<td>0.90 ± 0.82</td>
</tr>
</tbody>
</table>

Explanation in Tab. 1.
n – number of mice in each group = 10
EU – equivalent units to international units (determination by ELISA method)

The antigen content of the vaccines determined by in vitro ELISA method was lower than the antigenic values of vaccines determined by the LPL method (Table 5). The difference was significant with the adjuvant vaccine. The vaccine antigens were confronted with rabies antibodies levels after immunization (Table 5).

Table 5. Comparison of Rabicell vaccine efficacy evaluation and of live and inactivated rabies vaccine by in vivo (LPL) and in vitro (ELISA) methods

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Live vaccine</th>
<th>Inactivated vaccine</th>
<th>Commercial adjuvant vaccine Rabicell</th>
</tr>
</thead>
<tbody>
<tr>
<td>**Immunogenic activity (LPL)**n</td>
<td>ED₅₀ [mm²]</td>
<td>1.49 x 10⁻³</td>
<td>1.59 x 10⁻³</td>
</tr>
<tr>
<td></td>
<td>IA [IU/cm³]</td>
<td>1.876</td>
<td>1.759</td>
</tr>
<tr>
<td><strong>Antigen content</strong> (ELISA) [EU/cm³]</td>
<td>1.288</td>
<td>1.173</td>
<td>0.826</td>
</tr>
<tr>
<td><strong>Rabies antibody level</strong> (ELISA) [EU/cm³]</td>
<td>0.875</td>
<td>0.968</td>
<td>1.05</td>
</tr>
</tbody>
</table>

ED₅₀ – effective dose 50 %
IA – immunogenic activity
* – content of antigen in vaccine
** – rabies antibody titres on 21st day after immunization corresponding to LPL method
IU – international units
EU – units equivalent to international units

The potential use of the simplified ELISA test for the quantification of antigen content by means of diagnostic polyclonal rabies antibodies was recognised for live or inactivated non-adjuvant vaccines. The test is not suitable for the evaluation
of adjuvant vaccines. The antigen content of live and inactivated, but not adjuvant rabies vaccines, was in correlation with their immunogenic and antigenic activity. The antigen content of 1.0 EU/cm³ is considered the lower limit in sufficiently effective vaccines when using the ELISA method of determination.

**DISCUSSION**

The potency test for rabies vaccines developed in 1953 by the National Institute of Health (NIH) (Seligmann, 1973) is practically the only method that is currently used for the evaluation of efficacy of rabies vaccines (WHO, 1996), even though some manuals already permit alternative methods for the evaluation of inactivated vaccines (Europäische Arzneibuchkommission, 1997). Although the NIH method is considered to be a suitable method for rabies vaccines efficacy evaluation, it has been critiqued by a number of authors (Crick and Brown, 1974, 1978; Bijlenga, 1978; Aubert and Blancou, 1981; Bruckner et al., 1988; Cussler et al., 1998; Wunderli et al., 2003a; 2003b) in association with several questionable procedures used by this test. The test uses two vaccination doses on days 0 and 7. It is necessary to remember that a vaccine booster dose can mask the test results (Aubert et al., 1985). In addition to that the intraperitoneal immunization and intracerebral challenge route are unnatural (Barth et al., 1988). Consideration of adequate (natural) routes of immunization as well as of challenge (infection) of target animals are the most important criteria for objectivization of rabies vaccines efficacy evaluation tests in model experiments on laboratory mice (Crick and Brown, 1978; Wunderli et al., 2003a, 2003b). Experiments of Wunderli et al. (2003a) showed, that the vaccination route and the number of doses significantly influence the specific antibody response of mice. The protection against the challenge is independent of the vaccine strain origin. Challenge experiments (intracerebral administration of challenge virus) suggested the highest antibody answer in mice after intramuscular vaccination.

In our experiments the highest value of immunogenic activity was achieved with Rabisin tested by the NIH method, however, it did not correlate with the antigenic activity of this vaccine when intraperitoneal immunization was carried out. The results obtained with Rabicell were similar. The highest correlation rate between immunogenic and antigenic activities of the vaccines was detected with the LPL method, when the subcutaneous route of immunization and challenge was used. Otherwise the i.m./i.m. method (intramuscular vaccination and challenge route) of vaccine immunogenic activity evaluation was in correlation with antigenic activity values, overall the low values of both for the tested vaccines did not give a premise of adequacy of this method of injection rabies vaccines testing. The fourth way of evaluation (i.d./i.m.) appeared non-prospective for the same reasons as the previous one; maybe due to unpractical route of vaccine administration (into the nose tip). According to results of our experiments the most prospective method of in vivo evaluation of injection vaccines efficacy appears to be the LPL method (Švrček and Vrtiak, 1980), i.e. subcutaneous vaccine administration (into the upper shoulder area) with subcutaneous challenge.
In this study we performed experiments in order to investigate the possibility of a utilization of simplified ELISA test for the quantification of rabies vaccine antigen content (equivalent to antigenic value – AV, determined by in vivo method and calculated according to Reed and Muench, 1938) by diagnostic polyclonal rabies antibodies (hyperimmune sera). The antigen content of live and inactivated rabies vaccines was in correlation with their immunogenic and antigenic activities except for the commercial adjuvant (aluminium hydroxide) vaccine Rabicell which showed discordance between immunogenic and antigenic activities and antigen content. Similar results were obtained by Gamo et al. (1996) and Rooijakkers et al. (1996a). On account of this fact the simplified ELISA test apparently is not suitable for the evaluation of efficacy of adjuvant rabies vaccines as the adjuvant, whether aluminium hydroxide or a lipid one, can cause disproportionate results. Moreover, it is also necessary to consider the fact, that the in vitro method allows one to determine the whole content of antigen in the vaccine but not his immunogenic “quality” because successful immunization requires the antigen to be in a form comparative with the "natural" antigen. Therefore, the in vitro evaluation by simplified ELISA test seems to be appropriate for the evaluation during vaccine production. Thus, the unsuitable batches could be excluded from the production process. In addition, this could have an ethical impact as it reduces the number of laboratory animals necessary for vaccine efficacy testing.

Acknowledgement: This study was supported by projects VEGA 1/0572/03, 1/0574/03 and APVT-20-043902 (Slovak Republic).

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ISPITIVANJE EFIKASNOSTI INJEKCIJONIH VAKCINA PROTIV BESNILA – ALTERNATIVE

SÜLI J, BENIŠEK Z, ŠVRČEK Š, ONDREJKOVÁ A i ONDREJKA R

SADRŽAJ

Ova ispitivanja su izvedena sa ciljem da se racionalno i objektivno proceni in vivo efikasnost vakcine protiv besnila. Autori su ispitivali i uticaj načina aplikacije vakcine (subkutanog, intramuskularnog i intradermalnog) kao i efekte različitih načina probnih infekcija koji odgovaraju prirodnim putevima (subkutan i intramuskularni). Ispitivana je i korelacija između antigenosti i imunogenosti vakcina. Pri tome je ustanovljeno da je NIH metod nepouzdan, a da se visok stepen korelacije registruje LPL metodom. Za utvrđivanje imunogenog potencijala vakcine na osnovu koncentracije antigena, autori su koristili uprošćeni ELISA test. Ova metoda se može koristiti samo za vakcine bez adjuvansa. Dokazano je da se kao minimalna efikasna koncentracija antigena u vakcini može smatrati 1.0 EU/cm³.