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Reduction of the number of mice used for potency testing of human and animal rabies vaccines

L. Bruckner, M. Palatini, M. Ackermann, H. K. Müller and U. Kihm

Federal Vaccine Institute, Hagenaustrasse 74, CH-4025 Basel (Switzerland)

Summary. Eight different rabies vaccines were tested for their potency in the standard mouse potency test using 3-, 5- and 7-week-old mice. 5-week-old mice seem to be best suited for this purpose, variability from test to test could be reduced considerably. An ELISA was used in parallel for the evaluation of the rabies glycoprotein content of rabies vaccines. Results of the mouse potency test correlated well with those of the ELISA if highly purified human vaccines were tested. Unspecific reactions in the ELISA caused by adjuvanted veterinary vaccines could not be blocked. Further experiments will be needed in order to evaluate the potency of inactivated veterinary rabies vaccines by a in vitro test.
Key words. ELISA; mouse test; potency; rabies; vaccine.

Introduction

The goal of any vaccine potency test is the evaluation of the protective activity of the vaccine. The most direct and reliable way to evaluate the potency of a vaccine is the demonstration of its capability to protect the species for which the vaccine is intended, e.g. a rabies vaccine must be able to protect humans or dogs against a rabies virus infection. In this test, which is not feasable in humans, vaccination is followed by experimental infection.

For many years laboratory animal testing has been used, as an alternative, to evaluate the potency of vaccines. Induced immunity may be checked by infection as in the target species. Laboratory animals, compared to the host species, have the advantage of easy handling and housing; they can be produced in great number, so that experiments can be conducted in a quantitative way, and finally, they are relatively cheap. Tests on laboratory animals

have also inconveniencies: a correlation to results in the host species has to be established, and the number of parameters that can influence the test are numerous.
In contrast to animal tests, in vitro tests (usually physicochemical tests) do not evaluate the protective efficiency of a vaccine; they never include the reaction of an immune system. With in vitro tests, a specific protein which acts as an antigen is analyzed. Results of physico-chemical test procedures are more reproducible than those on animals. However, even though the in vitro results may be within the limit of acceptance, the vaccine may fail to induce an appropriate immune-response, due to various factors. Such tests are acceptable, if a correlation with protection tests in the host species exists.
In conclusion, any vaccine has to demonstrate its efficacy in the species it is intended for. For routine batch control either tests in laboratory animals or in vitro tests can be accepted if they correlate with the potency of the vaccine in the target species.

Potency control of inactivated rabies vaccines

It is well known that high rabies antibody titers protect against rabies virus infection[6]. The rabies glycoprotein has been found to be responsible for the induction of such neutralizing antibodies[3]. In the NIH-test immunization of mice is followed by a rabies virus challenge[5]. It consumes a lot of mice and reproducibility is poor. The NIH-test is the reference test for the estimation of rabies vaccine potency which is used by the World Health Organization, the European Pharmacopoeia as well as the Pharmacopoea Helvetica ed. VII[7–9]. Although correlation studies between results in the target species and NIH-test are scarce[2], the NIH-test is generally used. Several in vitro tests, i.e. the single radial immunodiffusion test (SRID)[4], the antibody binding test[1] and others are well established. Unfortunately most veterinary vaccines, as final products, are adjuvanted, and therefore these tests cannot be applied.
In our study we tried to improve the NIH-test in such a way that the test could be more reproducible, and we evaluated an enzyme-linked immunosorbant assay (ELISA), an in vitro test, for the potency testing of human and veterinary rabies vaccines.

Materials and methods

All NIH-tests were modified by using one single vaccination; each mouse was infected with 30–300 MLD_{50} i.c. of CVS. For each vaccine 4 groups with ten mice each were used. Ibm: MORO (SPF) 3-, 5- and 7-week-old mice were used.
The following rabies vaccines were used: PM strain WHO standard vaccine (TCO); PM strain European Pharmacopoeia standard vaccine (TCO); LEP strain purified concentrated vaccine (TCO); PM strain purified concentrated vaccine (TCO); SAD strain vaccine (TCO); CVS strain vaccine (NTO); PM strain purified concentrated vaccine (NTO).
For the ELISA the following material was used: 96 well microtiter plate (Dynatech M129B); PBSA (pH 7.4); 0.1 M Carbonate/bicarbonate buffer (coating buffer) (pH 9.6); ELISA wash PBSA + 0.1% Tween 20; ABTS-indicator solution (pH 4.2); 250 mM H_2O_2; 37 centigrade dry and moist incubator; rabbit anti-sheep-peroxydase conjugate; (Nordic Immunologicals RASh/IgG H + L)/PO); donkey anti-sheep-peroxydase conjugate (The Binding Site Ltd. Birmingham UK); sheep anti SAD-glycoprotein serum (M. Ferguson, NIBSC, London); skimmed mild powder; normal sheep serum; normal horse serum; normal mule serum; bovine serum albumine; gelatine.
For the ELISA the following method was used: Vaccines were diluted in coating buffer, plated intriplicate on the microtiter plate and left overnight in the dry incubator. After washing three times with ELISA wash, the plates were incubated with sheep anti-glycoprotein serum for one hour in a moist incubator and washed again. Conjugate was added for 1 h at 37 centigrade. Finally the indicator solution was added, and the optical density (OD) was read 20–30 min later. The principle of the ELISA procedure is shown in figure 1. The ELISA reader was connected to a personal computer for all further calculations.
The OD was plotted against the $\log_2$ of the dilution of the vaccine. The potency of the vaccine was estimated by referring to a standard vaccine in a parallel line assay by analysis of variance.

Results

The results of the different challenge tests are summarized in tables 1 and 2. The sensitivity of the mice (LD_{50}) is not influenced by the age of the mice used in the test. The PD_{50} shows a huge variation. Depending on the type of the vaccine used, the PD_{50} may be 3–60 times higher in 7-week-old mice compared to the 3-week-old mice. Unconcentrated nervous tissue origin vaccines (the old-fashioned vaccines) show the lowest rate of variation.

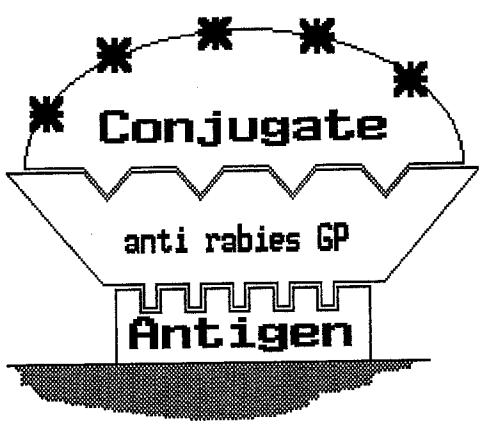

Figure 1.

Table 1. NIH-test with different age of mice at time of vaccination

Type of vaccine	Age of mice at time of vaccination in weeks	PD_{50} (mean value of 3 NIH-tests)	Relative PD_{50} (compared to 3-week value)
Standard WHO	3	390	1
	5	3268	8
	7	4009	10
Standard EP	3	236	1
	5	1845	8
	7	2105	9
Tissue culture origin	3	347	1
LEP strain	5	5170	15
purified, concentrated	7	10900	31
Tissue culture origin	3	32	1
LEP strain	5	227	7
purified, concentrated	7	268	8
Tissue culture origin	3	56	1
PM strain	5	517	9
purified, concentrated	7	3443	61
Tissue culture origin	3	0.9	1
SAD strain	5	4.4	5
	7	6.3	7
Nervous tissue origin	3	85	1
CVS strain	5	288	3
	7	259	4
Nervous tissue origin	3	137	1
PM strain	5	1968	14
purified, concentrated	7	8604	63

Table 2. NIH-test with different age of mice at time of vaccination (results of individual tests)

Type of vaccine	Age of mice at time of vaccination in weeks	PD_{50}
Tissue culture origin	3	65
LEP strain	3	48
purified, concentrated	3	12
	5	255
	5	136
	5	359
	7	125
	7	295
	7	427
Tissue culture origin	3	125
PM strain	3	0.6
purified, concentrated	3	214
	5	880
	5	443
	5	517
	7	830
	7	12650
	7	3440

Generally the difference between 5- and 7-week-old mice was negligible. We could notify a much lower range of variation within different tests using 5- and 7-week-old mice compared to 3-week-old mice.

Figure 2 shows the result of an ELISA test with a vaccine without an adjuvant. The optical density depends in a linear fashion from the concentration of the coated vaccine. Table 3 shows the effect of different blocking steps before and after coating of the vaccine.

Discussion

The refinement of the NIH-test indicated in a clear way, that it is very important, to use mice of a suitable age for this test. Using 5- and 7-week-old mice led to test results with a much lower variation, than those obtained with younger mice. Most probably the immune system of 3-week-old mice is not so well developed as it is in older mice. Unfortunately it is not possible to reduce the number of mice used in the test. For the evaluation of the test every single mouse is important for the calculation of the PD_{50}. A reduction of the number of mice used in each test, would lead to results, which are much less precise. With the ELISA we tried to replace the NIH-test. Our results showed, that it is possible to use the proposed test procedure for the evaluation of the potency of rabies vaccines for human use, which are produced on tissue culture. These vaccines are highly purified and not adjuvanted. In contrast, rabies vaccine for use in animals are

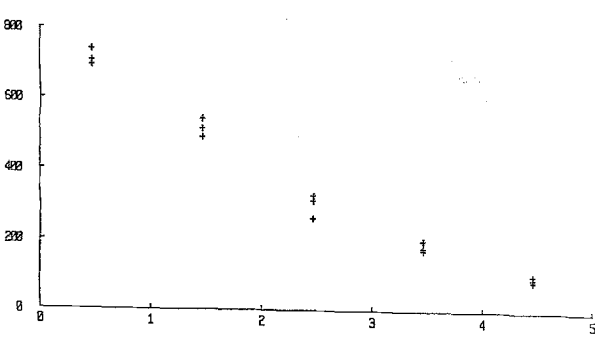

Figure 2.

Table 3. Effect of blocking of unspecific reactions of a rabies vaccine in the ELISA

		Vaccine without adjuvant		Vaccine with adjuvant	
		Complete ELISA	Conjugate control	Complete ELISA	Conjugate control
a) Blocking with different substances before coating of the vaccine onto the ELISA plate (liquid)					
10% skimmed milk powder		–	–	+++	+++
10% normal sheep serum		–	–	+	+
10% normal horse serum		–	–	+	++
5% normal horse serum		–	–	+	++
4% normal horse serum		–	–	+	++
3% normal horse serum		–	–	+	++
2% normal horse serum		–	–	+	++
1% normal horse serum		–	–	+	+
0.5% normal horse serum		–	–	+	+
30% bovine serum albumin		–	–	+	+++
30% bovine serum albumin		–	–	+	++
30% bovine serum albumin		–	–	+	++
30% bovine serum albumin		–	–	+	++
0.25% gelatine -		–	–	–	–
b) Blocking with different substances immediately after coating of the vaccine onto the ELISA plate					
10% skimmed milk powder		++++	++++	++++	++++
3% skimmed milk powder		++++++	+++	++++++	+++
1% skimmed milk powder		++++++	+++	++++++	++
0.3% skimmed milk powder		++++++	++	+	+
10% normal horse serum		++++++	+++	+++	+++
3% normal horse serum		++++++	+++	++	++
1% normal horse serum		++++++	+++	+	+
0.3% normal horse serum		++++++	+++	+	+
10% bovine serum albumin		++++++	++++	+++	+++
3% bovine serum albumin		++++++	+++	++	+
1% bovine serum albumin		+++	+++	+	+
0.3% bovine serum albumin		+++	++	–	–
c) Blocking with different substances in the anti-rabies-glycoprotein-serum and in the conjugate					
Block in the antiserum	Block in the conjugate				
5% normal horse serum	5% normal horse serum	–	–	+	+++
10% bovine serum albumin	10% bovine serum albumin	++++	++++	++++	++++
5% bovine serum albumin	5% bovine serum albumin	++++	++++	++++	++++
1% bovine serum albumin	1% bovine serum albumin	++	+++	+++	+++
0.5% bovine serum albumin	0.5% bovine serum albumin	+++	++	++	+++
0.1% bovine serum albumin	0.1% bovine serum albumin	+++	++	++	+++
0.05% bovine serum albumin	0.05% bovine serum albumin	+	+	++	+++
1% bovine serum albumin	1% bovine serum albumin	++	+++	++	+++
0.5% bovine serum albumin	1% bovine serum albumin	++	+++	++	+++
0.1% bovine serum albumin	1% bovine serum albumin	++	+++	++	+++
0.05% bovine serum albumin	1% bovine serum albumin	++	+++	++	+++
0% bovine serum albumin	1% bovine serum albumin	++	+++	++	+++

– no blocking effect; + poor blocking effect; ++ moderate blocking effect; +++ strong blocking effect; ++++ very strong blocking effect; ++++++ block covers all other reactions.

often adjuvanted with aluminium hydroxide and not purified to the same extent as human vaccine. Adjuvants and impurities in veterinary vaccines led to unspecific reactions in the ELISA test. We tried to block these undesirable reactions with different blocking steps. It was our goal not to block the rabies-specific reaction, and on the other side to get a complete block of the conjugate control. The best results were seen, if a blocking agent (normal horse serum) was added to the anti-glycoprotein-serum and to the conjugate. Even then, we could still see unspecific reactions. We assumed that these reactions are due to proteins, which were used during the production of the vaccine. Also, different conjugates led to different results. Conjugates produced in rabbits demonstrated an unspecific affinity towards rabies virus. The best results were obtained with a conjugate, which was produced in a donkey.

We can conclude that the ELISA is a suitable tool for the evaluation of the potency of rabies vaccines for human use, which are produced on tissue culture. The test has to be adapted for veterinary vaccine. We plan to further evaluate a double-sandwich-ELISA and a liquid phase ELISA for the potency evaluation of rabies vaccines.

List of abbrevations

ABTS = 2.2-Azino-di-(3-ethyl-benzthiazolin-sulfonate (6))-diammoniumsalt
CVS strain = rabies challenge virus standard
ELISA = enzyme-linked immunosorbant assay
HEP strain = high egg passage strain of rabies virus
LD_{50} = dose which kills 50% of inoculated animals

LEP strain = low egg passage strain of rabies virus
NTO = nervous tissue origin
OD = optical density
PBSA = phosphate buffered saline without Mg and Ca
PD_{50} = reciprocal of the dose which protects 50% of vaccinated animals
PM strain = Pitman-Moore strain of rabies virus
SAD strain = Street Alabama Dufferin strain of rabies virus
SRID = single radial immunodiffusion test
TCO = tissue culture origin
WHO = World Health Organization

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Robust regression in biological assay: application to the evaluation of alternative experimental techniques

A. Marazzi, C. Ruffieux and A. Randriamiharisoa

Institute of Social and Preventive Medicine, University of Lausanne, Bugnon 17, CH-1005 Lausanne (Switzerland)

Summary. Robust Huber type regression and testing of linear hypotheses are adapted to statistical analysis of parallel line and slope ratio assays. They are applied in the evaluation of results of several experiments carried out in order to compare and validate alternatives to animal experimentation based on embryo and cell cultures. Computational procedures necessary for the application of robust methods of analysis used the conversational statistical package ROBSYS. Special commands for the analysis of parallel line and slope ratio assays have been added to ROBSYS.
Key words. Biological assay; robust regression; validation of alternative assay techniques.

1. Introduction

The two major types of quantitative dose-response relations in biological assay are the *parallel line* and the *slope ratio* assay. Statistical analysis of these is based on multiple regression models and testing of linear hypotheses. Their basic assumptions and aims are summarized in Section 2, and a thorough discussion can be found in Finney[1].

Classical multiple regression analysis and linear hypotheses testing in quantitative bioassay are based on the method of Least Squares (LS, in the following) and the assumption that responses are independent random variables which are (for given doses) identically distributed according to the Gaussian (or normal) distribution. Under this assumption, the LS method is optimal (minimum variance and unbiased). However, the assumption of normality is often violated in reality. Dangerous departures from normality can be associated with the occurrence of gross errors, such as copying or keypunch errors or, more generally, occasional errors made when something indeterminate went wrong. The presence of anomalous subjects in the biological experimental material is a frequent reason for deviant responses. These departures usually show up as *outliers*, observations far removed from the majority of the data.

It is well known that the LS coefficient estimates in multiple regression are very sensitive to the presence of outliers; indeed, a single outlier can have an arbitrarily large effect on the estimates. Moreover, outliers typically inflate the standard error associated with estimates. As a consequence, the power of the classical F-test decreases drastically and the decision may completely change by removing a single distant point.

Example 1. In figure 1 a, the point P is an outlier. However, it is well aligned with the other points, and the LS