



REPORT OF WHO WORKSHOP ON NIH POTENCY TEST FOR RABIES VACCINES

Geneva, 4 - 6 November 1985

1. Introduction

During the last decade a number of inactivated animal rabies vaccines capable of inducing immunity of more than one year following a single inoculation have been developed and become widely available on the market. Prior to 1982 the increase in the duration of immunity conferred in the field by these new vaccine types (e.g. tissue culture and suckling mouse brain vaccines) had not been related to the vaccine potency as measured by the standard NIH mouse protection test in the laboratory.

Because of the divergency of inactivated vaccines (substrate, virus strain, inactivant, adjuvant) with different levels of potency in the NIH test and duration of protection in the field, WHO asked a number of vaccine producers to provide pertinent data on their products. In 1982 a group of consultants to WHO reviewed the information submitted by the vaccine producers on means or ranges of vaccine potency values of their vaccines. The WHO consultants recognized that additional pertinent information concerning the actual vaccine used for determining the duration of protection was necessary before an accurate assessment of the relationship between relative potency established by the NIH test and duration of protection could be made.

A Workshop on NIH Potency Test for Rabies Vaccines was held in Geneva, Switzerland, from 4-6 November 1985 to consider additional data from host animal immunogenicity tests and to determine if modifications to the NIH test might result in better correlation between relative potencies and the result of host animal protection tests. The list of participants is given in Annex 1.

2. Additional information on relative potency and host animal immunogenicity

The results of host animal immunogenicity tests of vaccine conducted at the National Veterinary Services Laboratories (NVSL), Ames, Iowa, USA, or conducted by vaccine manufacturers and submitted to the NVSL were analyzed to assess the relationship between relative potency and host animal efficacy. Data were available from three types of inactivated vaccine: tissue culture origin (TCO) Pasteur-derived vaccine, nervous tissue origin (NTO) Pasteur-derived vaccine, and TCO SAD strain vaccine.

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Table 1 gives the percent protection and percent of animals with antibody titers of  $\geq 20$  at the time of challenge for vaccine produced from TCO Pasteur-derived rabies virus. It is apparent that a relationship exists between relative potency obtained in the NIH test and protection for this type of vaccine. Pasteur-derived TCO vaccine had to contain 0.5 to 1.5 IU per dose before animals could be successfully immunized for one year with one dose of vaccine, and vaccine that immunized for three years generally contained  $> 2.5$  IU per dose. Thus the statement in the Report of the Seventh WHO Expert Committee on Rabies that vaccines with a two-year duration of protection have a relative potency of at least 2.0 IU per dose is true for TCO Pasteur-derived vaccine.

The increases in percent protection and duration of immunity seen with TCO Pasteur-derived vaccine as the relative potency measured by the NIH test increased were not seen with NTO Pasteur-derived vaccine (Table 2). Nervous tissue origin vaccines successfully immunized animals despite low relative potencies. Conversely, the only vaccines not able to immunize animals for one year contained 1.5 to 2.5 and  $\geq 2.5$  IU per dose. The data indicate that, unlike the relationship with TCO Pasteur-derived vaccines, the relative potency of an NTO Pasteur-derived vaccine established by the NIH test is not a good indicator of a vaccine's ability to immunize the target species.

Table 3 gives the percent protection and percent of animals with antibody titers of  $> 20$  at the time of challenge for vaccine produced from SAD strain rabies virus. As with NTO Pasteur-derived vaccine there is very little relationship between relative potency and protection in the host animal. The percent of animals with antibody titers of  $\geq 20$  does, however, increase as the relative potency increases. It is apparent from the data presented that, except for TCO Pasteur-derived vaccine, the relative potency of inactivated rabies vaccine measured by the NIH test is not a good indicator of the immunizing capability of vaccine in the target species.

### 3. Evaluation of serological response and protection from challenge

The street rabies virus challenge results in dogs and cats and antibody titers from 1767 antibody determinations made at the time of challenge for vaccines with satisfactory potency are presented in Table 4. The data represent 147 titers from animals vaccinated with Flury strain vaccine, 919 titers from animals vaccinated with SAD strain vaccine, and 701 titers from animals vaccinated with Pasteur-derived vaccine. The data are further divided into killed virus (KV), tissue culture origin (TCO) SAD vaccine; KV, TCO Pasteur-derived vaccine; and KV, nervous tissue origin (NTO) Pasteur-derived vaccine.

Based on the information presented the group concluded that there were no significant differences in the antibody response or protection from challenge elicited by the three types of inactivated vaccines analyzed.

### 4. Antigenic differences among strains of rabies virus

The analysis with monoclonal antibodies specific for the glycoprotein antigen of rabies reveals differences in the reactivity pattern of fixed and street rabies virus strains.

The TCO Pasteur-derived PM strain and mouse brain propagated CVS strain virus used as challenge in the NIH test are nearly indistinguishable. However, they are easily distinguishable from the tissue culture propagated CVS-11, SAD and Flury strains. Higher protection rates are obtained in homologous cross protection studies in mice than in the heterologous vaccination challenge situation.

Strains of virus isolated from terrestrial animals are in general more closely related to virus strains used for vaccine production than strains isolated from bats. Vaccines currently in use are able to protect experimental animals (mice) against challenge with all rabies virus isolates; however, the amount of viral antigen necessary to induce protection may be higher in cases of viruses of bat origin.

Despite these differences in the mouse model there is no evidence that antigenic variations observed by analysis with monoclonal antibodies plays a role in the efficacy of rabies prophylactic vaccination, providing that the vaccine used is of adequate potency. This can be substantiated by the excellent protection record in the use of human diploid rabies vaccine in preventing rabies in man.

#### 5. Principles and variations in rabies vaccine potency tests

Different properties of a vaccine can be used to measure potency of inactivated rabies vaccines. They include induction of resistance to challenge, induction of virus neutralizing antibodies or cell mediated immunity, and in vitro estimation of glycoprotein content. Different tests measure these properties in different ways.

##### 5.1 Testing of resistance to challenge in the target species or in laboratory animals

An example for this kind of test is the NIH test. It is recommended by WHO as the standard potency test for inactivated rabies vaccines prepared from suckling mouse brain or cell culture material. The test has several disadvantages such as the length of time to perform the test (28 days), large variation in test results on the same vaccine, and its lack of correlation with the protective immunity produced in the target species by adjuvanted SAD origin vaccines and nervous tissue origin vaccines. Various modifications in the challenge strain, number of vaccinations, route of vaccination, and route of challenge have been examined and some modifications appear to correlate better with host animal protection.

##### 5.2 Testing virus neutralizing antibody production in target species or laboratory animals

These tests measure the ability of the antigen to induce antibodies and is applicable to all types of vaccine. Coorelation has been established between the antibody induction test in laboratory animals and the standard NIH test. However, short-term serology does not correlate with long-term protection in the target species.

##### 5.3 Testing for development of cell-mediated immunity

A lymphocyte blastogenesis assay has been used to measure cell-mediated immunity to inactivated rabies vaccines in dogs. Using the assay, 83% of the dogs vaccinated with a TCO SAD vaccine and 50% of the dogs vaccinated with either a TCO Pasteur-derived vaccine or an NTO Pasteur-derived vaccine responded with positive

stimulation indexes. The antigen used in the assay was SAD virus. The significance of these findings has not been fully evaluated but the ability of vaccine to induce a cell-mediated immune response may be an important factor in the pre-exposure immunization of animals.

#### 5.4 In vitro tests

Assays which have been applied to examine the rabies vaccine potency include antibody binding tests, single radial immunodiffusion (SRD) and enzyme immunoassays (ELISA). Such tests have the advantage that they are rapid and can be used for "in process" control. However, only ELISA can be used for adjuvanted vaccines. The ideal potency test should correlate with efficacy in the host animal, distinguish between high and low potency vaccines, and be easily standardized, practical and economically feasible. At present there is no test that meets all of these requirements.

#### 6. Recent developments in In Vivo testing

The WHO Expert Committee on Rabies (Seventh Report) recommended that vaccine with a relative potency of less than 1.0 IU per dose with the NIH test should not be approved unless an adequately designed experiment has demonstrated a duration of immunity of at least one year in the species for which the vaccine is to be used. However, it has become apparent that not all types of vaccines perform identically in the NIH test. This is especially true of SAD origin rabies vaccines which have been proven to be efficacious in the host animal but have low relative potencies in the standard NIH test. For this reason two collaborative studies were conducted to evaluate (i) modifications in the NIH test, and (ii) the CDC test as alternative in vivo potency tests.

##### 6.1 Modifications of the NIH test

The relative potencies of 12 inactivated and adjuvanted SAD origin rabies vaccines were evaluated using five modifications of the standard NIH mouse potency test. The five modifications were:

- one vaccination with CVS challenge at 14 days after vaccination;
- one vaccination with SAD challenge at 14 days after vaccination;
- two vaccinations 7 days apart with SAD challenge at 14 days after the first vaccination;
- two vaccinations 21 days apart with CVS challenge at 28 days after the first vaccination;
- two vaccinations 21 days apart with constant adjuvant (5% Aluminium hydroxide) in the diluent and CVS challenge at 28 days after the first vaccination.

All vaccines were tested by the standard NIH mouse potency test in six laboratories and these results were compared to the results obtained from each of the modified NIH mouse potency tests.

The single vaccination with CVS challenge 14 days after vaccination did not affect the relative potency. A single vaccination with SAD challenge 14 days after vaccination yielded an average relative potency value that was approximately 3-fold higher than the value obtained in the standard NIH mouse potency test. Two vaccinations with SAD challenge 14 days after the first vaccination resulted in an average 5-fold enhancement of the relative potencies of these vaccines. No change in vaccine potency was observed when the vaccination interval was extended to 21 days, and the use of adjuvanted diluent (5% Aluminium hydroxide) in the extended vaccination interval study lowered the relative potencies of the vaccines.

Results from these studies indicate that homology between the vaccine strain and the challenge virus was the single most important factor in increasing the relative potency of the inactivated and adjuvanted SAD origin rabies vaccines. The relative potencies of these vaccines were approximately five times higher in the standard NIH mouse potency test when the SAD strain of rabies virus was used as the challenge instead of the CVS strain. The enhancement resulted from an increased PD<sub>50</sub> of the SAD origin vaccine while the PD<sub>50</sub> of the Pasteur-derived reference remained constant.

It is felt that the use of the homologous challenge more accurately measures the true antigenicity of TCO SAD strain vaccines since it results in relative potencies that are in closer agreement with the relationship between IU and protection in the target species established for the TCO Pasteur-derived vaccines. Its use also results in relative potencies that are consistent with the WHO statement that vaccines with a two-year duration of protection have a relative potency of at least 2.0 IU per dose.

The introduction of the homologous SAD challenge in the NIH testing of SAD origin vaccine may be justified by the fact that the Pasteur virus-derived CVS virulent challenge was chosen as a homologous challenge at the time when all human vaccines were prepared from strains derived from Pasteur virus (CVS for duck embryo vaccine in the USA, suckling mouse brain vaccine in South America, PM or Pasteur virus in Europe and in other parts of the world.

## 6.2 The CDC test

The CDC test with its natural routes of peripheral vaccination and challenge (one intramuscular vaccination in the thigh followed 14 days later by intramuscular challenge with street rabies in the opposite thigh) has been used to test 20 rabies vaccines, including TCO SAD strain rabies vaccine, TCO Pasteur-derived vaccine, suckling mouse brain origin Pasteur-derived vaccine, and the 4th International Standard for rabies vaccine.

The CDC test results with these vaccines appear to be more reliable than the NIH test results in comparing vaccine types with comparable host animal efficacy. This may be due to an immunopotentiating effect of adjuvanted vs non-adjuvanted vaccine observed in the CDC test and not the NIH test. Additional replicate testing of vaccines of proven host efficiency is necessary to establish the reproducibility within and between laboratories.

The CDC test is practical and economically feasible. If it is shown to be reproducible and correlation to host animal protection results is superior to the NIH test, consideration should be given to replacing the NIH test with the CDC test.

## 7. Recent developments in In Vitro testing

### 7.1 Single radial immunodiffusion (SRD)

The SRD test has been shown to be a reproducible assay for the glycoprotein content of tissue culture origin rabies vaccines for human use. The effect of virus strain difference between reference and test vaccines on the relative potencies obtained in SRD assays has been investigated. Vaccines containing LEP and PM virus were tested in gels containing anti-ERA, anti-PM and anti-LEP glycoprotein serum. If test and reference vaccines were prepared from different strains of rabies virus considerable variation was observed among potencies obtained on gels containing different antisera. However, if reference and test vaccines were homologous the antiserum used in assays was irrelevant. The development of reagents for use in SRD assays of each strain of virus used in the production of tissue culture vaccines needs to be considered.

Samples of bulk material prior to addition of adjuvant of 10 of the 12 SAD vaccines included in the collaborative studies on modifications of the NIH test were assayed by SRD. Only one out of 10 vaccines contained sufficient glycoprotein to be easily assayed by SRD and four vaccines appeared to be of low potency, but were just assayable by SRD. Five vaccines were of too low potency to be assayed precisely by SRD. The potencies obtained from SRD assays appeared to correlate better with the potencies obtained in the modified NIH test in which the SAD strain was used as challenge virus than with the standard NIH test.

Although SRD assays of glycoprotein content do not provide direct evidence of immunogenicity, as does the NIH mouse protection test, they do give precise estimates of the amount of the major protective antigen of rabies virus in a vaccine. The SRD technique is reproducible and simple to perform. Its use for the potency testing of non-adjuvanted vaccine would reduce reliance on the imprecise in vivo test used at this time. In addition, SRD assays are of value in the "in process" control of vaccines by manufacturers and for evaluating the consistency of batches of vaccine.

### 7.2 Enzyme Immunoassay (ELISA)

These assays have been found suitable for measuring rabies virus antigens. One such procedure has been established and standardized in Essen. Briefly, vaccine dilutions are air dried on ELISA-microtiter plates followed by an incubation with a polyclonal anti-rabies virus protein serum. A second antispecies IgG serum coupled with peroxidase is then incubated and substrate added.

Approximately 100 different samples have been tested. These consisted of the WHO 1st - 3rd Reference Vaccines, substandards of companies, "in process" samples, concentrates, and final vaccines of various strains prepared on several substrates. Valid results which correlated well with results from the standard NIH test were obtained for all vaccines, including those adsorbed to  $Al(OH)_3$ . Vaccines as low as 0.01 IU could be measured in the assay.

## 8. Conclusions

Information presented at the Workshop confirmed that the NIH relative potencies of TCO Pasteur-derived vaccines can be related to protection in the host animal but NIH results of TCO SAD origin vaccine or NTO Pasteur-derived vaccine do not possess the same relationship. There are no apparent differences in the neutralizing

antibody response in the target species or the relationship between antibody titers and protection from challenge among these three types of inactivated vaccines. It was shown that vaccines of different types that respond equally in host animal protection studies may have significantly different NIH relative potencies. Therefore vaccines of different types can not be compared by NIH relative potencies alone.

Modifying the NIH test by changing to an SAD challenge increases the relative potency of SAD vaccines but does not affect the ED<sub>50</sub> of the CVS reference or relative potencies of the few Pasteur-derived vaccines that have been tested by this method. The relative potencies of TCO SAD origin vaccines obtained by this modification are comparable to relative potencies of TCO Pasteur-derived vaccines with corresponding host animal efficacy measured by the standard NIH test. The group felt that before changing to a homologous challenge in the NIH test, viruses other than CVS (street virus, SAD, LEP) should be evaluated as a possible challenge for all strains of vaccine.

The CDC test (single intramuscular vaccination and intramuscular challenge) produces higher relative potencies for TCO SAD vaccines and some TCO Pasteur-derived vaccines. It does not appear to increase the potency of NTO vaccines. Preliminary results obtained with the CDC test indicate it is superior to the standard NIH test in relating all types of vaccines to host animal protection studies.

In addition to the differences attributed to vaccine strains seen with in vivo test systems data were presented at the Workshop that demonstrated a strain effect on the in vitro SRD test. If test and reference vaccines were prepared from different strains of rabies virus considerable variation was observed among potencies on SRD gels containing different antisera. However, if reference and test vaccines were homologous the antiserum used in the assay was irrelevant.

#### 9. Recommendations

Based on the information presented at the Workshop the group made the following recommendations:

9.1 Since it is apparent that relative potencies obtained in the NIH test do not correlate well with the results of host animal protection studies for all types of vaccines, available data from host animal protection tests should be given priority when establishing minimum relative potencies for inactivated rabies vaccine.

9.2 Further collaborative studies should be conducted on modifying the challenge used in the NIH test and on the CDC test to determine if either assay system will correlate better with host animal protection. Representatives from all vaccine types (strain, substrate, adjuvant, etc) should be included.

9.3 The group recognized that in vitro tests such as SRD and ELISA play an important role in quantitating and standardizing the glycoprotein content of rabies vaccines used in man. Their usefulness in the testing of adjuvanted veterinary vaccines should be determined. Results of in vitro tests should be compared to results from in vivo potency tests and previous host animal protection tests. The reproducibility between laboratories of ELISA techniques should be examined.

Table 1: The Relationship between Relative Potency and Immunity for Tissue Culture Origin Pasteur Derived Inactivated Rabies

International Units	Number of Dose	Year Challenged	Percent Protection	Percent Titer $\geq 20$
$<.1$	1	1	33	0
$<.1$	2	1.5	50	25
$<.1$	2	1	$\frac{70}{\bar{x}}$	$\frac{0}{8}$
$>.1 - .5$	2	1.5	56	22
$>.1 - .5$	1	1	60	0
$>.1 - .5$	2	1	$\frac{74}{\bar{x}}$	$\frac{6}{9}$
$>.5 - 1.5$	1	1	69	44
$>.5 - 1.5$	1	1	70	15
$>.5 - 1.5$	2	1	70	40
$>.5 - 1.5$	1	1	83	13
$>.5 - 1.5$	1	3	89*	0
$>.5 - 1.5$	2	1.5	$\frac{90}{\bar{x}}$	$\frac{20}{22}$
$>1.5 - 2.5$	1	1	100	15
$>1.5 - 2.5$	1	1	$\frac{100}{\bar{x}}$	$\frac{42}{29}$
$>2.5$	1	3.5	100	40
$>2.5$	1	3.5	100	50
$>2.5$	1	3	100	73
$>2.5$	1	3	100	83
$>2.5$	1	1	100	92
$>2.5$	1	1	100	100
$>2.5$	1	3	100	100
$>2.5$	1	3.5	100	100
$>2.5$	1	3.5	$\frac{100}{\bar{x}}$	$\frac{100}{82}$

\*Mortality in controls was <80%

Table 2: The Relationship between Relative Potency and Immunity for Nervous Tissue Origin Pasteur Derived Inactivated Rabies

International Unit	Number of Dose	Year Challenged	Percent Protection	Percent Titer $\geq 20$
$\leq .1$	2	1	90	0
$>.1 - .5$	1	1	90	0
$>.1 - .5$	2	1	$\frac{100}{95}$	$\frac{20}{10}$
			$\bar{X}$	
$>.5 - 1.5$	2	1	87	25
$>.5 - 1.5$	1	1	95	23
$>.5 - 1.5$	1	1	96	82
$>.5 - 1.5$	1	1	100	42
$>.5 - 1.5$	1	1	100	60
$>.5 - 1.5$	1	1	$\frac{100}{96}$	$\frac{100}{55}$
			$\bar{X}$	
$>1.5 - 2.5$	1	1	79	0
$>1.5 - 2.5$	1	3	90	7
$>1.5 - 2.5$	1	1	94*	84
$>1.5 - 2.5$	1	1	96	0
$>1.5 - 2.5$	1	1	100	8
$>1.5 - 2.5$	1	3	100	52
$>1.5 - 2.5$	1	1	$\frac{100^*}{94}$	$\frac{91}{35}$
			$\bar{X}$	
$>2.5$	1	1	61*	7
$>2.5$	1	3	93	61
$>2.5$	1	1	100	8
$>2.5$	1	3	$\frac{100}{89}$	$\frac{26}{26}$
			$\bar{X}$	

\* Mortality in controls was <80%

Table 3: The Relationship between Relative Potency and Immunity for SAD Strain Inactivated Rabies

International Unit	Year Challenged	Percent Protection	Percent Titer $\geq 20$
<.1	3	84	0
<.1	3	92	0
<.1	1	92	16
<.1	1	96	20
<.1	1	96	23
<.1	3	96	25
<.1	1	100	96
		$\bar{X}$ 94	26
>.1 -.5	1	76	42
>.1 -.5	1	84*	19
>.1 -.5	1	93	70
>.1 -.5	1	94	6
>.1 -.5	1	97	27
>.1 -.5	1	100	29
>.1 -.5	1	100	86
>.1 -.5	1	100	93
		$\bar{X}$ 93	47
>.5 -1.5	1	88	0
>.5 -1.5	3	92	80
>.5 -1.5	3	100	61
>.5 -1.5	1	100	69
>.5 -1.5	3	100*	89
		$\bar{X}$ 96	60

\*Mortality in controls was <80%

Table 4: Challenge Results from Rabies Immunogenicity Test  
with  $\geq 87\%$  Protection  
(Death/Challenged)

Vaccine	Antibody Titer *				
	<5	5-9	10-19	20-39	$\geq 40$
Flury	1/50	0/16	0/26	0/15	0/40
SAD	26/211	5/99	2/157	0/114	1/338
Pasteur	18/177	3/107	5/111	1/66	0/240
SAD (KV, TCO)	21/156	2/63	1/116	0/79	0/150
Pasteur (KV, NTO)	13/133	2/62	5/73	1/34	0/164
Pasteur (KV, TCO)	5/44	1/45	0/38	0/32	0/76

\* Reciprocal 50% end points determined by either the mouse neutralization test or RFFIT.

ANNEX 1

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\*Invited, but unable to attend.