ACTA VET. BRNO, 49, 1980: 259–267

APPLICABILITY OF SHOPE FIBROMA VIRUS REPLICATED IN CELL CULTURES FOR IMMUNOPROPHYLAXIS OF RABBIT MYXOMATOSIS

J. JEŘÁBEK

Department of Prophylaxis of Pig Diseases, Animal Breeding and Zoohygiene, University of Veterinary Science, 612 42 Brno

Received April 13, 1979

Abstract

Jeřábek J.: *Applicability of Shope Fibroma Virus Replicated in Cell Cultures for Immunoprophylaxis of Rabbit Myxomatosis.* Acta vet. Brno, 49, 1980: 259–267.

Shope fibroma virus replicated on cell cultures of RK 13 line is suitable for the preparation of lyophylized vaccine against myxomatosis in rabbits. The vaccine is safe and its efficiency is satisfactory. After a single subcutaneous application good immunity may be expected for next three months. Revaccination provides further extension of immunity with 75 % of vaccinated population still immune seven months later.

Myxomatosis, vaccination, Shope fibroma virus, in vitro cultivation.

In principle, two types of vaccines have been employed for the immunoprophylaxis of myxomatosis in rabbits: 1. Vaccines prepared from Shope fibroma virus (SFV) have been used since the discovery of rabbit immunity against myxomatosis after the application of this virus (Shope 1932; Shope 1936a; Shope 1936b; Shope 1938; Rowe, Mansi and Hudson 1956; Jacotot, Vallée and Virat 1958; Roemmele 1958; Durand et al. 1974; Matthes 1978). 2. Vaccines prepared from the attenuated myxomatosis virus (Anonymous 1970; Jiran, Sladká and Kunstýř 1970; Saurat, Gilbert, Ganière 1978).

It follows from the literature to date that none of the vaccine types reported can ensure permanent immunity after a single or revaccination followed vaccination (Eissner 1966; Durand et al. 1974; Loquerie, Rayon, Durand 1977; Saurat, Gilbert, Ganière 1978; Eissner, Weber 1978). To achieve satisfactory immunity against myxomatosis it is thus advantageous to work out a suitable vaccination scheme rather than choosing one or another type of vaccine. When attenuated myxomatosis virus is applied the question remains to be solved concerning possible circulation of the vaccination virus in rabbit population (Anonymous 1973).

So far, Shope fibroma virus (SFV) for the vaccine preparation has been replicated in rabbits (Mádr, Macura, Petlach 1967; Mádr 1971; Jacquemont et al. 1972; Anonym 1973).

Although there are reports on SFV replication facilitated in different cells *in vitro* (Kilham 1957; Kilham 1959; Verna, Eylar 1962; Hinze, Walker 1964; Israeli, Sachs 1964; Woodroofe, Fenner 1965; Hinze, Walker 1971; Tektoff, Gazzolo, Leftheriotis 1971; Jacquemont et al. 1972) the possibility of use of such virus for the vaccination purposes has not yet been considered. This report describes the results concerning vaccine preparation from *in vitro* replicated SFV.

Materials and Methods

Cell cultures

Taking into account that SFV may be successfully cultured in cells of rabbit origin (Jacquemont et al. 1972) we employed the RK 13 cell line of rabbit kidney. Two types of growth media have been tested: Earle medium and MEM (Eagle) medium. Both media were supplemented by 10 % of fetal bovine serum for cell cultures. Because the Earle medium proved to be suitable

only up to the fourth passaging of RK 13 cell line exclusively the MEM medium was used for further replications. Using the latter medium it was possible to obtain the cell monolayers suitable both for virus passaging and for the actual vaccine preparation. As the medium for culture maintenance after the infection of cell monolayer the Earle as well as MEM media were used, none of them containing bovine serum.

Culture passaging of RK 13 cell line

The subpassages of RK 13 cell line were prepared from well developed cultures preserved in Roux bottles, usualy after a week's intervals. After the removal of medium the culture was washed with the versen-trypsin solution at 37 °C, then rinsed and immersed for 5 minutes into versen--trypsin. After this time the supernatant was removed and the bottles were turned so that their culture containing parts were facing up. In this arrangement they were placed into a thermostat at 37 °C for 20 minutes. When the residual versen-trypsin was decanted the free cells were mixed to form a suspension in the MEM growth medium. Out of one Roux bottle contents usually 3 vessels of the same culture volume were obtained.

Virus

The starting strain for the adaptation to the RK 13 cell line the commercial vaccine against rabbit myxomatosis was selected. This vaccine is prepared from SFV replicated on rabbits and it has been commercially produced by Bioveta at Ivanovice na Hané.

Challenge virus

To check the immunity after the SFV application the dose corresponding to 1000 ID_{50} of virulent myxomatosis virus was applied upon carrying out the preliminary experiments (Jeřábek unpublished results) which revealed no difference in the course of myxomatosis after the challenge by 10^2-10^5 ID_{50} of virulent myxomatosis virus in rabbits. The overall challenge dose (1000 ID_{50}) was administered into both conjunctival sacs (one drop each), the rest (up to 1 ml) being applied subcutaneously.

Rabbits

To make sure that both vaccine and the virus passaged on RK 13 cell line are safe and efficient, healthy animals weighing 1.5—2.0 kg were selected out of breeds where no previous vaccination against myxomatosis was carried out.

Procedure of virus passaging

Three-day-old cell monolayers of RK 13 cell line were used for the infection by virus. These monolayers were prepared in two ways:

1. The same procedure as that applied for passaging cultures of the RK 13 cell line
2. The same as above, the only difference being the use of Earle medium.

This second possibility may be employed only for the preparation of culture which is to be used for the infection by virus. As already mentioned the Earle medium cannot be used for a routine passaging of RK 13 cell line cultures.

Virus passaging

The developed monolayer of RK 13 cells was infected by the storage virus (i. e. the suspension of virus from the previous passage) and after a 30 minutes' adsorption the contents of Roux bottle were filled with the nine-fold amount of medium (3 cm^3 of virus and 27 cm^3 of medium were applied in the case of 300 cm^3 bottles, 5 cm^3 of virus and 45 cm^3 of medium for 500 cm^3 bottles and 10 cm^3 of virus and 90 cm^3 of medium for 1000 cm^3 bottles). In case that the RK 13 line cells were cultivated prior to infection in the MEM growth medium, the maintenance MEM medium was added. When the Earle medium was used for replication of cells to be infected, the medium for maintenance according to Earle was also used. The adaptation of virus as well as its other passage on the RK 13 cell line was thus carried out parallely in MEM and Earle medium.

After filling up the medium the cultivation of the virus proceeded in a stationary manner at 37 °C. When a significant cytopathic effect occurred (4th post - infection day) on the infected cells the latter were subjected to three freeze and thaw cycles. The suspensions obtained in this way represented the virus which was used for further passage. Efficiency and safety were verified in rabbits with the tenth and twentieth passage.

The vaccine preparation

The virus obtained from the tenth passage of RK 13 cell line was used for the actual vaccine preparation. Procedures of monolayer preparation and infection were identical with that of virus passage. After three freeze-thaw cycles the contents of individual Roux bottles were joined and the

suspension thus obtained was stored at −20 °C. Upon thawing, the suspension was mixed with the liquid lyophilic medium (0.5 ml of medium per 1 ml of suspension). The composition of medium was following: gelatine for bacteriology 300 g; sucrase 450 g; $Na_2HPO_4.12H_2O$ 22.92 g; KH_2PO_4 2.92 g; redistilled water 6000 cm³. After the lyophilization and the sterility control tests were carried out establishing vaccine efficiency and safety. Invariably 6 cm³ were taken for lyophilization; these contained 4 cm³ of virus suspension and 2 cm³ of lyophilic medium. Twenty vaccination doses were obtained upon dilution of the lyophilizate with 20 cm³ of diluent. The latter was either MEM and Earle medium, or buffered saline.

The efficiency and safety of vaccine were tested according to procedure adopted in Czechoslovakia for rabbit myxomatosis vaccine prepared from SFV replicated in rabbits.

After the control tests had been completed the production of the lyophilized vaccine has started up under the commercial name SFT rabbit myxomatosis vaccine (SFT standing for Shope Fibroma virus replicated in Tissue cultures).

Safety test

Four experimental rabbits were observed for health status upon application of vaccine.

Procedure

Rabbits with shaved backs were employed. Each animal was given 3 cm³ of commercial vaccine intradermally by 12 injections à 0.25 cm³. Another two animals were given subcutaneous dose (3 cm³ each) applied on two separate sites (behind shoulder blade).

Evaluation of the test

The experimental animals are observed for 21 days. In intradermally inoculated animals pea-or bean-sized fibroma must develop at the site of application of vaccine but no generalization of fibromatosis may occur. Subcutaneously vaccinated animals must not exhibit general fibromatosis or more serious local changes.

A small local fibroma occurring at the site of application is not considered serious. It is usually absorbed without detrimental effects before the end of observation period.

Apart from the above - mentioned test the safety of vaccine was checked by the subcutaneous application of vaccine to young animals after weaning.

During the safety and efficiency testing of SFT vaccine in rabbits also the pure culture RK 13, containing no SFV, was inoculated intradermally (similarly to procedure applied for the virus titration). Two rabbits were given the suspension in MEM while in the other two cases the Earle medium was used. No reaction was observed upon the intradermal application. Upon challenge the animals developed myxomatosis and died.

The test of vaccine efficiency

and of the passaged virus was made by determining ID_{50} according to Reed and Muench (1938) after the titration on rabbits. Moreover, challenge followed upon the application of passaged virus and one vaccination dose, respectively. The titres of virus in individual passages and in the prepared vaccine were compared with that of virus (vaccine) replicated in classical way in rabbit skin.

Virus titration

The titrations of harvested virus and lyophilized vaccine were always carried out by intradermal testing on the shaved backs of two experimental animals. Each rabbit was given 0.25 cm³ of the undiluted vaccine or virus suspension. Moreover, corresponding dilutions down to 10^{-5} were administered. Each dilution was applied by eight injections at the rate of 0.25 cm³. The reaction was evaluated from 6th to 12th day after the application. The size of developed fibromas was measured. The skin thickness was subtracted from the observed value in mm. The increase in thickness of 1 mm and more was considered positive for the calculation of ID_{50}.

Comparing ID_{50} of individual virus passages or vaccines it was possible, according to titres, to evaluate their efficiency but the experimental values did not quite reflect the intensity of reaction of individual tests. Therefore a complementary criterion was adopted and referred to as overall reaction number (ORN) which represents an average of all daily sums (from 6th to 12th day) of all thickness increases in mm (size of fibromas) upon the application of individual dilutions of the virus being studied. The comparison of ORN corresponding to individual passages or vaccines contributes to a better comparison of their effectiveness.

The determination of protective doses in one vaccination dose (PD_{50})

Although the comparison of ID_{50} and ORN of individual passages of virus or vaccines enables to compare their relative effectiveness, the immunogenic effect of the vaccination dose is not sufficiently expressed i. e. it is not known how many protective doses are present in one vaccination

Table 1

Average logarithms of ID_{50} and average ORN of in cell cultures passaged Shope Fibroma Virus and prepared SFT vaccine

Tested	Original virus	10th passage	10th passage	1 10/0	2 x 10/0	2 10/0	2 10/0	2 xx 10/0	2 xx 10/0	20th passage	20th passage
Maintenance medium	–	MEMo	Eo	Eo	Eo	Eo	Eo	Eo	Eo	MEMo	Eo
Diluent	Eo	MEMo	Eo	Eo	H_2O Eo	Eo	PFR	Eo	PFR	MEMo	Eo
log ID_{50}/0.25 cm³	4.3	3.5	3.8	3.4	3.6	3.8	3.4	3.7	3.5	3.5	3.5
ORN in mm	233	169	249	228	262	246	187	294	208	188	210

Eo = Earle medium without serum
MEMo = MEM medium without serum
PFR = buffered saline
1 10/0 = titration of virus used for the vaccine preparation before lyophilization
2 10/0 = titration of the lyophilized vaccine
x = lyophilizate diluted to original volume with distilled water, further dilution effected by Eo medium
xx = titration of vaccine 24 hours after dilution, diluted vaccine stored at +4 °C

Table 2

Safety and effectiveness of the passaged virus and the SFT vaccine

Tested	10th passage	10th passage	20th passage	20th passage	Lyophilized vaccine	Lyophilized vaccine	Lyophilized vaccine	Lyophilized vaccine	Lyophilized vaccine	Lyophilized vaccine
Maintenance medium	MEMo	Eo	MEMo	Eo	Eo	Eo	Eo	Eo	Eo	Eo
Diluent					Eo	PFR	Eo	PFR	Eo	PFR
Applied	0.5 cm³ s. c.	0.5 cm³ s. c.	1 cm³ s. c.	1 cm³ s. c.	1 v. d. (= 1 cm³) s. c.	1 v. d. (= 1 cm³) s. c.	3 v. d. (= 3 cm³) s. c.	3 v. d. (= 3 cm³) s. c.	3 v. d. (= 3 cm³ = 12 × 0.25 cm³) i. d.	3 v. d. (= 3 cm³ = 12 × 0.25 cm³) i. d.
Number of rabbits	1	1	2	2	8	2	2	1	2	1
Reaction after application	0	0	0	0	0	0	0	0	fibromas at application sites	fibromas at application sites
Challenge carried out upon the application	11th day	11th day	10th day	10th day	9th day	9th day	–	–	–	–
Result of the challenge	0	0	0	0	0	0	–	–	–	–

v. d. = vaccination dose, s. c. = subcutaneously, i. d. = intradermally, 0 = without reaction, – = not tested

dose. Therefore the number of protective doses (PD_{50}) was determined by means of titration on rabbits. To determine their value the calculation by Reed and Muench (1938) was employed.

The lyophilized virus suspension was diluted to the original volume (= 10°) with redistilled water used for the preparation of tissue culture media. Further dilution was effected using the Earle medium for culture maintenance. The groups of experimental animals were given the individual dilutions of vaccine, the dose being 1 cm^3 each. After 19 days of application the rabbits were infected with 1000 ID_{50} of virulent myxomatosis virus. After the challenge the rabbits were observed for 6 weeks. Two standpoints were considered for the determination of PD_{50}: 1. Only those animals were considered to be surviving for the determination of PD_{50} which exhibited no reaction upon the challenge; 2. As surviving animals also those were considered which developed a reaction at the site of inoculation (primary myxoma) or temporary myxomatosis (benign myxomatosis).

The test of effectiveness by challenges of vaccinated rabbits at various time intervals after a single vaccination and after vaccination followed by revaccination

The vaccination dose of 1 cm^3 was always used for both the vaccination and revaccination of rabbits. The revaccination was done 25th day after the first vaccination. The challenge was effected at various times after vaccination (revaccination) using the usual dose of myxomatous virus; the rabbits were then observed for 21 days.

In all tests of effectiveness of passaged SFV and of the vaccine prepared from it always two control rabbits were infected by the same challenge dose which upon challenge developed myxomatosis and died or were killed.

Results

Titration of passaged virus and of prepared vaccine

Table 1 indicates that when compared with the original virus titre the titres were lower by 0.5—0.9 logs and that the ORN is not always directly dependent on log ID_{50}. It is further obvious that the maintenance medium Eo is suitable for virus replication as well as a diluent of lyophilized vaccine. This fact is important from the economical point of view as well. Also buffered saline can be used for dilutions. The decrease of diluted vaccine effectiveness did not occur during 24 hours if both maintenance medium Eo and buffered saline were used. It is also evident from the Table that during passaging neither log ID_{50} decrease nor ORN decrease occurred. Moreover, none of the followed values was negatively influenced by the lyophilization.

Assay of safety and of effectiveness of passaged virus and prepared vaccine

Upon the subcutaneous application of various doses of passaged virus and prepared vaccine no postvaccination reaction was observed (Table 2). Upon intradermal inoculation only fibromas at the inoculation site occurred. All rabbits, which had been challenged by virulent myxomatous virus, on the 9th—11th day after the subcutaneous application of passaged virus or vaccine, survived without reaction. Although no groups of the same number of experimental rabbits were used it is evident from Table 2 that no difference was found when two different diluents were used, i. e. Earle maintenance medium and buffered saline.

Apart from the results presented in Table 2 the safety and effectiveness of prepared vaccine were tested on young animals from two not vaccinated females.

From one mother three young rabbits were vaccinated immediately after weaning at the age of 23 days by the dose of 1 cm^3 of diluted lyophilized vaccine (determined vaccination dose). No postvaccination reaction was observed. On the 45th day after vaccination the rabbits were infected by 1000 ID_{50} of virulent

myxomatous virus. Apart from a slight temporary infiltration at the inoculation site in two rabbits no further symptoms of the disease were noted.

From the other female rabbit two youngs aged 29 days (also immediately after weaning) were each vaccinated by a 3 cm^3 dose of diluted lyophilized vaccine, this dose being triple of that used in the previous experiment. No reaction after the vaccination was observed. Challenge carried out 34th day after the vaccination did not induce any reaction except a slight temporary infiltration at the inoculation site in one rabbit.

Number of protection doses (PD_{50}) in 1 cm^3 of SFT vaccine

To determine PD_{50} the SFT vaccine containing $10^{3.8}ID_{50}/0.25$ cm^3 of lyophilized virus suspension, as found by titration on rabbits (see Table 1) was used.

Data to be used for the PD_{50} calculation are presented in Table 3. Since the commercial SFT vaccine consists of lyophilizate (equal to 4 cm^3 of virus suspension and 2 cm^3 of lyophilic medium) which is diluted by 20 cm^3 of diluent prior to vaccination, 1 cm^3 of diluted vaccine, that is one vaccination dose, contains 12.6 — 400 PD_{50} ($4 \times 63.1 = 252.4 : 20 = 12.6$; $4 \times 2000 = 8000 : 20 = 400$).

Table 3

Determination of PD_{50} in 1 cm^3 of SFT vaccine

Dilution	Number of rabbits	a) Reaction to challenge negative	a) Reaction to challenge positive	b) Reaction to challenge negative	b) Reaction to challenge positive
10^{-6}	4	0	4	0	4
10^{-5}	4	1	3	1	3
10^{-4}	4	1	3	3	1
10^{-3}	3*	1	2	2	1
10^{-2}	4	1	3	2	2
10^{-1}	3*	1	2	1	2
10^{0}	4	4	0	4	0
PD_{50}		$= 10^{-1.8}/1$ cm³		$= 10^{-3.3}/1$ cm³	
PD_{50} contained in one vaccination dose		12.6		400	

a) Only those rabbits were used for the determination of PD_{50} which did not exhibit any reaction after the challenge
b) To determine PD_{50} rabbits exhibiting no reaction and those with the benign myxomatosis upon challenge have been used
* The fourth rabbit from the experimental group died for other reason

The immunity duration in rabbits with single vaccination and in rabbits vaccinated and revaccinated by the same SFT vaccine dose on the 25th day after the first vaccination

The result of immunity duration studies after a single vaccination are given in Table 4. Up to the 84th day after a single vaccination the rabbits did not react upon challenge by virulent myxomatosis virus. In rabbits infected within a longer time interval after the vaccination the reaction induced by challenge was more distinct and apart from myxomatosis with benign course a typical myxomatosis with subsequent death developed.

The results of immunity duration studies after vaccination followed by subsequent revaccination are summarized in Table 5. It is evident that up to the 121st

Table 4

The immunity of rabbits vaccinated subcutaneously using 1 cm³ of SFT

Number of rabbits followed	Challenge 1000 ID_{50} of virulent myxomatous virus effected upon the vaccination	Result of challenge			
4	36th day	BN	BN	BN	BN
3	84th day	BN	BN	BN	
4	116th day	BN.	BN.	BM	BM
4	146th day	BN	BM	BM	BM
4	183rd day	BN	BN •	BM	M
4	210th day	BN	BN –	M	M
3	253rd day	BN –	BM	M	

BN no myxomatosis symptoms
BN. no effect, only local temporary reaction at the inoculation site of myxomatous virus
BN – only temporary increased breathing and congestion of conjuctiva
BM myxomatosis reaction followed by a subsequent recovery recovery (benign course of myxomatosis)
M myxomatosis with a typical course, animal died or was killed

Table 5

Immunity of rabbits subcutaneously vaccinated and revaccinated with 1 cm³ of SFT vaccine 25 th day after the first vaccination

Number of animals followed	Challenge 1000 ID_{50} of the virulent myxomatous virus carried out after revaccination	Result of challenge			
4	59th day	BN	BN	BN	BN –
2	102nd day	BN	BN.		
4	121st day	BN	BN	BN	BN –
4	158th day	BN	BK	BN.	M
4	185th day	BN	BN	BN	M
4	228th day	BN	BN	BN –	BN –

day after revaccination all rabbits survived the challenge without a marked reaction. The challenges effected from 158th to 228th day (no experiments with longer period have been conducted) after the revaccination 75—100 % of rabbits survived without more distinct reaction.

Comparison of data presented in Table 4 and 5 shows that better immunity is obtained in rabbits vaccinated and revaccinated.

Discussion

It follows from hitherto available data on immunoprophylaxis of myxomatosis that no vaccine exists guaranteing a long-lasting immunity after single vaccination or after vaccination followed by revaccination in a vaccinated rabbit population. This fact is important mainly for breeding animals where vaccination scheme providing a satisfactory immunity has to be chosen for a period of myxomatosis peak occurrence.

Present results show that the SFT vaccine prepared from SFV replicated on monolayer of RK 13 cell line yields at least the same effect as vaccines against rabbit myxomatosis at present produced abroad.

The replication of virus in a defined standard cell substrate and in a maintenance medium guarantees the preparation of safe and effective vaccine. The use of Earle maintenance medium for virus multiplication and of the same medium or buffered saline as a diluent of lyophilized vaccine is advantageous also from the economical point of view.

By orientation testing on young animals and by experiments on mature rabbits it has been established (Matthes 1978) that the vaccine prepared from the SFV is safe for rabbits of various age categories. The subcutaneous vaccination is not followed by postvaccination reactions. This fact confirms the data by Eissner (1966) who found that the immunity was not dependent on the fibroma development at the site of inoculation. In one vaccination dose (equal to 1 ccm) 12.6 — — 400 PD_{50} is contained.

When vaccination with subsequent revaccination were employed practically 100 % immunity was found four months after the revaccination. Even seven months after revaccination (experiments with longer time period have not been undertaken) the immunity was found to be solid in at least 75 % of vaccinated population. If a single vaccination was effected the same degree of immunity could be expected approximately for three months. It follows from the above stated data that by vaccination with subsequent revaccination using SFT vaccine in spring months a solid immunity in rabbits could be ensured for the rest of the year.

Použitelnost viru Shopeho fibromu pomnožovaného na buněčných kulturách k imunoprofylaxi myxomatózy králíků

Virus Shopeho fibromu pomnožovaný na buněčných kulturách linie RK 13 je vhodný k přípravě lyofilizované vakcíny proti myxomatóze králíků, která je neškodná a dostatečně účinná. Po jednorázové podkožní aplikaci vakcíny je možno počítat se solidní imunitou po dobu tří měsíců. Po vakcinaci s následnou revakcinací je možno ještě za sedm měsíců počítat se solidní imunitou nejméně u 75 % vakcinované populace.

Применяемость вируса Фибромы Шоупа, разможенного на клеточных культурах, для иммунопрофилактики миксоматоза проликов

Вирус фибромы Шоупа, размноженный на клеточных культурах линии РК 13 удобен для подготовки лиофилизированной вакцины против миксоматоза кроликов, которая безвредна и достаточно действенна. После однократного подкожного ввода вакцины можно положиться на возникновение устойчивого иммунитета в течение трех месяцев. После вакцинации с последующей повторной вакцинацией можно еще спустя семь месяцев положиться на устойчивый иммунитет минимально в 75 % вакцинированных кроликов.

Acknowledgement

The author thanks Ing. L. Šimková for technical assistance.

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