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PRODUCTION IN VERO CELLS OF AN INACTIVATED RABIES VACCINE FROM STRAIN FRV/K FOR ANIMAL AND HUMAN USE

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Summary. — A new concentrated and purified rabies vaccine was produced in Vero cells. Two rabies virus strains, the fixed rabies virus Pasteur (FRV) and Pittman Moore (PM) were adapted to Vero cells by 20 cycles of alternating passages in the brain of weaning mice. Intracerebral (i.c.) inoculation of weaning mice was followed then by 17 and 20 serial passages in Vero cells of RFV and PM strains, respectively. The adapted strains designated as FRV/K and PM/K gave titres of $10^6 \pm 1.5$ log (LD_{50}/ml for i.c. inoculated mice) in several harvests taken from one infected cell culture. Pooled harvests were concentrated 20-fold by ultrafiltration and were tested as animal vaccine after inactivation with beta-propiolactone (BPL). Another vaccine preparation destined for human use, in addition to concentration and inactivation, was also purified by gel filtration. Control tests revealed that the antigenic content of different strain FRV/K harvests was very high in comparison with that of strain PM/K and the reference tissue culture vaccine (RIV, Netherland). In sheep the antibody response induced by the FRV/K strain was very high; serum neutralizing index (NI) higher than 4 was reached 40 days after the second vaccine dose, whereas the vaccine preparation from strain PM/K gave NI of 2.3 and the reference vaccine NI of 3.8, respectively. Safety tests in rabbits and guinea pigs showed neither pyrogenicity nor toxicity.

Key words: inactivated rabies vaccine, cell cultures, production, antigenic potency and safety tests

Introduction

In Egypt and many other developing countries, the Fermi type rabies vaccine (1908) is produced in brain of young goats or sheep. This vaccine has been used until now for post-exposure treatment of man while for animal immunization the LEP and HEP rabies vaccines were preferred.

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Different types of rabies virus tissue culture vaccines have been introduced and are extensively used in the U.S.A. and Europe because they lack side effects and cause no neuroparalytic complications like the Fermi type vaccine.

Koprowski (1967) demonstrated that WI-38 human diploid cell was the substrate of choice for the production of rabies virus vaccine in tissue culture. The Wistar strain of rabies adapted to human diploid cells has been used by manufacturers in many countries for vaccine production. Subsequently Wiktor *et al.* (1973) endorsed the use of this vaccine in the U.S.A. The human diploid cell adapted Wistar strain is used now by manufacturers in U.S.A., France and Netherlands for vaccine production.

Another tissue culture rabies vaccine for human use was developed by Selimov and Aksenova (1966). This vaccine was produced in primary Syrian hamster kidney cells infected by Vnukovo-32 strain of rabies virus and used for pre- and post-exposure treatment in the U.S.S.R. and Eastern Europe since 1967. In Netherlands, van Wezel and van Steenis (1978) described the large scale production of a purified, inactivated rabies vaccine in primary dog kidney cells cultivated on microcarriers; the vaccine has been used for post-exposure treatment of man in this country (in our experiments it is used as a reference vaccine).

Further tissue culture rabies vaccine was developed by Berlin *et al.* (1982) who used Rhesus diploid cell cultures for production of a rabies vaccine adsorbed onto aluminium phosphate gel. The vaccine given in two doses for post-exposure treatment in man induced an excellent rabies antibody response in volunteers. Recently, a new inactivated tissue culture rabies vaccine for use in man (purified chick embryo vaccine) was developed in Federative Republic of Germany (Barth *et al.*, 1984).

In the presented work based on our preliminary report (El-Karamany, 1984), Vero cells were chosen as substrate for rabies vaccine because of the cell growth potential in microcarrier culture and the high virus yield, i.e. conditions suitable for an economical large scale production of inactivated rabies vaccine in developing coutries. In the meantime, the Vero cell rabies vaccine was recommended also by other authors (Montagnon *et al.*, 1985).

Materials and Methods

Rabies virus strains used were the Pittman Moore (PM) and the Fixed rabies (FRV, Original Pasteur strain, passage 12 in rabbit brain), employed in our laboratory for production of Fermi type vaccine in goat brain.

Cell culture. Vero cells Flow lab, England, were supplied from the tissue culture unit (Virus Research Centre, Cairo).

Animals. Weaning mice, 21-day-old, rabbits, guinea pigs and sheep were supplied from laboratory animals farm (Helwan).

Adaptation of rabies strains to Vero cells. Adaptation of the 2 rabies virus strains to Vero cells was done by alternating passages in the mouse brain and Vero cells. One passage in Vero cells was followed by 3 to 5 passages in the mouse brain; this cycle was repeated till the titre of the virus reached its optimum level again. Then the virus was passed in Vero cells once followed in mice 2 to 3 times, increasing the number of passages in Vero cells and decreasing the number of passages in mice. After 20 of such alternating passages, the virus was serially passaged in Vero cells for up to 17 (strain FRV) and 20 passages (strain PM), respectively. The adapted rabies virus strains were designated as PM/K and FRV/K (K = Karamany).

Rabies virus assays. After each passage in Vero cells the rabies strains were tested by direct immunofluorescence (Goldwasser and Kissling, 1958) to calculate the percentage of infected cells. To determine the infectivity in every 3rd passage of both rabies virus strains adapted to Vero cells, the tissue culture fluid harvests were titrated in mice by i.c. inoculation. Ten-fold dilutions from 10^{-1} to 10^{-7}. were inoculated into 10 mice each by i.c. route; the mice were observed for 21 days for paralyses or death. The LD_{50} values were calculated by the method of Reed and Muench (1938).

Rabies virus antigenicity was assayed by ELISA technique carried out according to van der Marel and van Wezel (1981). Antirabies dog immunoglobulin was used for coating plates, the reference vaccine, antirabies sheep immunoglobulin, anti-sheep peroxidase conjugate and substrate were kindly supplied by Dr. van Wezel, RIV (Netherlands).

Preparation of the vaccine. Vero cells-adapted rabies virus strains were propagated in Vero cells grown either on microcarriers (strain PM/K) or as monolayers in plastic flasks (both PM/K and FRV/K strains). In the former case Vero cells adjusted to contain 100×10^3 cells/ml were grown in 3 liters culture volume fermentor containing 2.5 g of cytodex 3/L medium at temperature of 34 °C, pH 7.2, and oxygen tension of 50 % air saturation. Cells formed confluent monolayer on the beads after 3 to 5 days in culture reaching the concentration of $1-2 \times 10^6$ cells/ml in 5 days. In one culture Eagle's MEM with 2 % Ultroser G (serum-substitute), in another Eagle's MEM with 10 % foetal calf serum were used. The PM/K virus strain was used after 16 passages in Vero cells (CP 16) to infect the cells; multiple harvests were taken each 5 to 6 days replacing the spent culture fluid with serum-free fresh media until the cell culture on the microcarrier beads was exhausted. Virus harvests were stored at +4 °C, small samples being kept at −70 °C.

In the latter case, Vero cells were grown to confluency in Corning plastic (650 ml) bottles. Then the growth medium was removed, the cells were washed 3 times each with 50 ml phosphate buffered saline (PBS) and the seed virus was inoculated. After 1 hr virus adsorbtion, 100 ml medium M 199 without serum were added to each bottle and the bottles were incubated at 34 °C. All virus-infected cell cultures were daily observed. The first harvest was collected from infected cultures on days 4−6 post-infection (p.i.) and kept at +4 °C. Then 100 ml fresh medium was replaced to each flask and further incubated. Multiple harvests were obtained after 5 to 7 days when cytopathic changes of the cells were clearly observed and the final extract harvest (cell-free and cell-associated virus) were obtained by 3 times freezing and thawing. Small samples taken from each harvest were kept at −70 °C to be titrated in mice for infectivity and to be tested by ELISA for antigenicity.

Virus concentration, purification and inactivation. The clarified virus suspensions were pooled and concentrated by ultrafiltration through Amicon Hollow fiber filters H 10×100 (cut off 100,000) at +4 °C. All lots were concentrated from 20 to 40 times. The concentrated virus was purified by gel filtration on a DEAE-Sepharose 6B-CL column under conditions when the cellular DNA binds to the gel but the virus is eluted. The virus was titrated in mice and tested by ELISA for recovered antigen content. Finally, the virus was inactivated by BPL at a final concentration of 1 : 4,000.

Control tests. All the experimental vaccine lots prepared during this study were subjected to tests according to the WHO requirements for safety, innocuity and antigenic potency (WHO Tech. Rep. Ser, 1981). Habel and NIH tests for potency were carried out according to the procedures described by Habel (1973) and Seligman (1973). In addition, all separate harvests plus samples taken from the pooled harvests from one culture, ultrafiltrate, concentrated virus, purified and inactivated final product fractions were tested for antigenic content by ELISA in comparison with the reference tissue culture inactivated RIV vaccine (Netherlands) prepared in primary dog kidney cells and having the potency of 3.1 international untis (IU). Optical density (OD) values obtained with two-fold dilutions (from 1.10 to 1.640) of materials tested were recorded and their antigenic content was calculated according to the formula $\frac{a}{b} \times 3.1$, where 'a' means dilution of product tested giving OD = 0.700 and 'b' refers to dilution of the reference RIV vaccine with the same OD.

Immunogenic potency was tested in subcutaneously (s.c.) inoculated sheep by the assay of virus neutralizing antibodies following immunization with the two adapted PM/K and FRV/K rabies virus strains in comparison to concentrated reference and purified RIV vaccines. The virus neutralizing index (NI) was obtained by substracting the log of the LD_{50} titre of the 10-fold

dilutions of rabies virus against postvaccination serum from that against pre-vaccination serum (Johnson, 1973).

Safety tests for toxicity and pyrogenicity were carried out in guinea pigs and rabbits which were inoculated s.c. with 0.5 and 1 ml of vaccine preparation, respectively. The body temperatures were recorded daily for up to day 21 p.i.

Results

Propagation of rabies virus strains PM/K and FRV/K in Vero cell monolayers

Vero cell monolayers grown in plastic flasks were infected with PM/K (CP20) and FRV/K (CP17) rabies virus strains at multiplicity of infection (MOI) of 0.01. Altogether 4 harvests of either virus strain were collected at 5—7 days intervals starting from days 4—6 p.i. to determine the virus titre and the antigen yield. As shown in Table 1, the highest titre of PM/K virus strain was found in the 1st harvest taken on day 6 p.i., whereas the higher yields of virus antigen were obtained at later p.i. intervals. The indices found with PM/K strain, however, did not exceed those of FRV/K at all intervals tested (except the virus titre from the 1st harvest), the highest levels of the latter strain being observed at the 2nd harvest on day 9 p.i. Similar results, i.e. higher titre and antigen yield of FRV/K than PM/K strain, were also obtained when testing other Vero cell passages of these rabies virus strains.

Antigenic content and potency of the vaccines produced from strains FRV/K and PM/K in comparison with the reference RIV vaccine

PM/K (CP20) and FRV/K (CP17) virus strains harvested from Vero cells were purified by gel filtration and their antigenic content as determined by ELISA was compared with that of the reference RIV vaccine. In parallel,

Table 1. Propagation of rabies virus strains PM/K and FRV/K in Vero cell monolayer culture

Virus strain (CP)	Harvest no.	Harvest day	Titre (log LD_{50}/ml)[a]	Virus antigen yield (units/ml)[b]
PM/K (20)	1	2	7.02	0.48
	2	13	4.85	1.16
	3	19	4.52	0.89
	4	26	4.52	0.4
FRV/K (17)	1	4	6.85	2.4
	2	9	7.02	6.46
	3	14	5.02	5.8
	4	19	4.52	0.48

[a] — Determined in mice by i.c. inoculation
[b] — determined by ELISA
CP = cell passage
Antigenic content of harvests 2 and 3 of strain FRV/K was higher than that of the reference vaccine (NI 3.1, 1 IU)

Table 2. Antigenic content and potency of two purified rabies vaccines produced from the two Vero cells-adapted strains PM/K and FRV/K in comparison with a reference RIV vaccine

Vaccine strain	Antigen content (units/ml)	NIH test	Habel test*
PM/K	0.9	1.8	1500
FRV/K	4.06	9.4	5000
Reference RIV vaccine	3.1	3.1	n.t.

* Protection against the virus dose (mouse LD_{50})
n.t. — not tested.

vaccine potency was determined using NIH and Habel tests. As follows from Table 2, antigenic content was much higher in purified vaccine product from FRV/K than from PM/K virus strain, surpassing even the level of 3.1 IU of the reference RIV vaccine. Similarly, vaccine prepared from FRV/K strain was of higher potency both in NIH and namely in Habel test, in which it afforded protection of mice against 5,000 LD_{50} of rabies virus, while the vaccine from PM/K strain against 1,500 LD_{50} only. In this connection it is worth mentioning that the WHO requirement of a potent rabies virus vaccine is based on protection against 1,000 LD_{50} in Habel test and ELISA antigenic content above 3.1 IU.

Seroconversion in sheep immunized with vaccines prepared from the two Vero cells-adapted rabies virus strains PM/K and FRV/K or with the reference RIV vaccine

Sheep were s.c. inoculated with two doses (22 days apart) of BPL-inactivated purified vaccine prepared from Vero cells-adapted rabies virus strains PM/K (CP20) and FRV/K (CP17) or with the reference RIV vaccine. Their blood was collected on days 10, 21, 31 and 62 starting from the 1st day of vaccination, i.e. on days 9 and 40 following the 2nd vaccine dose and NI

Table 3. Seroconversion in sheep immunized with vaccines from the two Vero cell-adapted rabies virus strains PM/K and FRV/K or with reference RIV vaccine

Vaccine strain	$\log_{10}$ NI value on days post-vaccination			
	10	21	31(9)*	62(40)*
PM/K	0.3	0.5	1.7	2.3
FRV/K	1.3	1.5	2.5	≥4
Reference RIV vaccine	1	1.5	2.5	3.8

* In parentheses days after the 2nd vaccine dose.
Vaccine produced from strain FRV/K was immunogenic in sheep, NI in sheep vaccinated with two doses being more than 4 (neutralization of more than 10,000 LD_{50} of challenge virus).

Table 4. Virus titre and antigenic content in crude, purified and concentrated preparations of rabies virus strain PM/K propagated in Vero cell microcarrier culture

Harvest no. or further product	Harvest day	Cell concentration[a]	Virus titre (log LD_{50}/ml)[b]	Antigen content (units/ml)[c]
1	5	1,950	7.01	0.49
2	9	600	6.52	0.7
3	12	310	6.68	0.2
4	16	60	5.52	0.89
Pooled harvest			n.t.	0.7
Filtered harvest			n.t.	0.3
Concentrated virus 30-fold			7.86	3.1
Ultrafiltrate			negative	negative
Concentrated + BPL inactivated			negative	2.0
Final purified product + lactose			negative	0.9

[a] — $\times 10^3$/ml
[b] — upon i.c. inoculation to mice
[c] — by ELISA
n.t. — not tested

was determined in serum pooled from 5 animals at each post-inoculation interval (Table 3).

Vaccine prepared from FRV/K strain was of the highest immunogenicity at all post-vaccination intervals tested. In sera of sheep immunized with two doses of this vaccine and collected on day 62 post-vaccination, the NI was higher than 4 (neutralization of more than 10,000 LD_{50} of the challenge virus inoculated to mice) comparing to NI values of 3.8 and 2.3 in sheep sera collected at the same interval post-vaccination with reference RIV vaccine and vaccine prepared from PM/K strain, respectively.

Safety tests

No pyrogenic and toxic reactions either in rabbits or in guinea pigs inoculated s.c. with the vaccine lots prepared from PM/K and FRV/K virus strains occurred within 21 days observation period indicating that the vaccines produced fulfilled the WHO requirements for safety.

Virus titre and antigenic content in crude, purified and concentrated preparations of rabies virus strain PMK propagated in Vero cell microcarrier culture

In further experiments, the virus titre and antigen yield were followed in the series of harvests from Vero cells grown in microcarrier culture.

In addition, the yield of virus antigen was determined in a pool of 4 harvests after filtration, concentration and inactivation. It can be seen in Table 4 that a slight decrease of the virus titre with increasing number of virus harvests was accompanied by a decrease of the cell concentration, but not by a decrease of the yield of virus antigen. The latter dropped in the pooled harvest after filtration, but increased again after 33-fold concentration, which also resulted in an increase of the virus titre. Inactivation of this concentrated product by BPL led to further decrease of its antigenic content and the final purified product enriched by lactose contained even less antigen. However, overall infectivity and antigenicity of PM/K virus harvests from Vero cell microcarrier culture were higher when compared with the same indices of this strain grown in the Vero cell monolayer culture (Table 1). As follows from the results of preliminary experiments (data not presented), the same can also be applied to the FRV/K virus strain.

Discussion

Need for a safe, economic and potent rabies virus vaccine prevails, especially for post-exposure human vaccination. Several tissue culture adapted rabies vaccines are commercially available (Selimov and Aksenova, 1966, Wiktor *et al.*, 1973; van Vesel and van Steenis, 1978, Berlin *et al.*, 1982; Barth *et al.*, 1984). Yet, as stated in WHO recommendations, trails at the use of a more convenient cell substrate for rabies vaccine should be pursued. Hereby, we present data on two rabies virus strains PM/K and FRV/K adapted for large scale production to the growth in Vero cell culture. As follows from them, compiled from the results of potency tests according to international standards (WHO tech. rep. serv., 1981), strain FRV/K proved to be superior to the other PM/K strain and appeared to have some merits over the reference RIV vaccine. It applies not only to the virus titre in harvests collected at different intervals p.i., but especially to the antigenic yield obtained, on which also the potency and immunogenicity of vaccine tested was shown to be depended.

The virus titre and antigenic content could be increased by propagating the Vero cells-adapted rabies virus strains in Vero cells grown on microcarriers rather than in monolayer culture. Further experiments are required, however, to determine the suitability of microcarrier culture use for large scale vaccine production. To increase the yield of rabies antigen in a vaccine product, concentration of pooled virus harvests by ultrafiltration through amicon Hollow fiber filters was proved efficient. The product obtained and inactivated by BPL can be used as animal vaccine. For human use, the concentrated and inactivated vaccine product should be purified further by gel filtration on DEAE-Sepharose 6B-CL.

All the experimental vaccine lots met the WHO requirements for safety (WHO Tech. Rep. for 1981), namely non-pyrogenicity and nontoxicity in rabbits and guinea pigs. Work is going on for the establishment of further safety tests before putting new vaccine for human trials.

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