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Production methods for rabies vaccine

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A concise history of rabies vaccine production methods, including worldwide production methods in animals and cell culture are presented.

Keywords: rabies vaccines; production methods

Rabies, a viral disease that affects all warm-blooded animals, is widespread in many regions of the world. It is a subject of great concern because of its propagation among wildlife, such as foxes, wolves, skunks, raccoons, bats, mongoose and, obviously, dogs, which behave as a permanent reservoir for the virus.

The first rabies vaccine successfully employed in humans, was developed by Louis Pasteur *et al* in 1885 [75,76]. Administering several doses of a suspension of desiccated rabbit marrow extracted from an animal infected with rabies virus, Pasteur managed to cure a young shepherd who had been bitten by a rabid dog. Since then, several types of vaccine have been developed and employed.

First generation vaccines

First generation vaccines are all produced using an animal substrate to obtain a viral mass [43]. These vaccines have been widely used in humans as well as in animals. Table 1 shows the main features of first generation vaccines. Considering the kind of substrate employed in their production, first generation vaccines may be classified as follows:

Vaccines produced by employing adult animal nerve tissues

Vaccines based on adult animal nerve tissues are not unlike the first vaccine developed by Louis Pasteur. However, numerous complications are observed, namely:

- Presence of residual live virus: vaccines such as the Fermi vaccine [29,62], which contain live virus because of deficient inactivation with phenol, are considered mixed vaccines (residual live virus and inactivated virus) and should not be used.
- Post-vaccinal encephalomyelitic reactions: certain vaccines from this group including the Semple [91,97] and Hempt vaccine [38] types are fully inactivated but, despite fulfilling potency requirements, severe post-vaccinal encephalomyelitic reactions have occurred during or after treatment. Reactions are caused by the *encephalitogenic factor* (a basic protein associated with myelin) arising from adult animal nerve tissue employed as substrate. Reactions involve neuroparalytic accidents likely to range from a slight temporary paresthesia to a permanent neurological injury, and even death [10,37,52,66,106,107].
- Low antigen content per dose of vaccine. This feature made long treatments necessary with a great number of inoculations. These vaccines, generally sold in a liquid vehicle, had low stability of approximately 6 months.

Vaccines manufactured with embryonated eggs

An adaptation of rabies virus strains in duck embryos [7,39,69,81,123] (Pitman Moore strain) enabled the development of much safer rabies vaccines for human use, diminishing neuroparalytic post-vaccinal encephalomyelitic reactions.

The incidence of neurological reactions is much lower (less than three per 100 000 treatments) in human vaccines produced in duck embryos than in those produced in nerve tissue. However, local reactions are quite common, reaching 70% in prophylactic vaccination and 100% in complete, 14-dose rabies treatments [86]. Immunogenicity of this vaccine type is a matter of debate due to the low antibody level provided in some cases [20,26,34,53,79,98,99,108]. Flury and Kelev fixed virus strains were adapted to chick embryo through repeated passaging (180 for Flury and 70 for Kelev), yielding attenuated viral strains [50]. Vaccines produced with attenuated virus in chicken embryos are used in dogs, cats and cows.

Very high quality embryonated eggs must be free from all kinds of adventitious virus (aviary leukosis) and bacteria (salmonella) for use in vaccine production.

Vaccines produced in suckling animal brain

The encephalitogenic factor [40,42,112] likely to be responsible for post-vaccinal neurological accidents is present in adult animal nerve tissue, but is negligible or absent in certain suckling animals. Together with the higher viral productivity achieved in new-born animal nerve tissue, this finding has fostered development of vaccines employing suckling animals [33,104]. The first vaccine of this type was developed by Fuenzalida and Palacios [32] in suckling mouse brain, employing three strains of fixed rabies virus

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Table 1 Main features of first generation rabies vaccines for human and veterinary use

Substrate employed	Virus strain	Vaccine type	Inactivation method	Human use	Pharmaceutical form	Reference
NT: sheep, goat	Pasteur	Inactivated	Phenol (1 day)	Yes	L	[29]
NT: rabbit, sheep, goat	Pasteur	Inactivated	Phenol, 30°C	Yes	L	[97]
NT: sheep	Pasteur	Inactivated	Ether-Phenol	Yes	L	[38]
NT: sheep	Pasteur	Inactivated	Phenol (14 days)	Yes	FD	[95]
NT: sheep	PV11	Inactivated	βPL	Yes	FD	[62]
Suckling mouse brain	CVS, 51, 91	Inactivated	UV	Yes	L	[32]
Suckling rabbit brain	PV	Inactivated	UV	Yes	FD	[33]
Suckling rat brain	PV	Inactivated	Phenol (14 days)	Yes	FD	[104]
Duck embryo	PM	Inactivated	βPL	Yes	FD	[81]
Chicken embryo	Flury LEP	Attenuated	–	No (only for dogs)	FD	[50]
Chicken embryo	Kelev	Attenuated	–	No (only for dogs)	FD	[50]
Chicken embryo	Flury HEP	Attenuated	–	No (cattle, cats)	FD	[50]

NT: Nervous tissue; FD: freeze-dry; L: liquid; βPL: β-propiolactone; UV: ultraviolet radiation; LEP: low egg passage; HEP: high egg passage.

(CVS, 51 and 91). Strains are inoculated in 3-day-old mice and 4 days later rabies symptoms become apparent, when brain mass is harvested. This vaccine is employed in all Latin American and several African countries as well. It is employed in humans (pre- and post-exposure) and in animals.

A substantial improvement has been achieved at the Instituto Nacional de Microbiología Dr Carlos G Malbrán of Buenos Aires, Argentina, consisting in changing the substrate to employ suckling rats instead of mice [90] (according to Kabat *et al* [42] and Svet-Moldavskij *et al* [104]). Only one-day-old animals are employed to minimize myelin content in the viral mass harvest, a process allowing a high viral productivity (10^6–10^7 LD_{50} in mice) to be achieved with shorter harvest times (3 days post inoculation) and a higher infected mass (50% higher than in mice) without increasing hazards caused by the encephalitogenic factor [22]. Finished vaccine consists of a viral suspension (diluted to 1% for human use, and to 2.5% for veterinary use) inactivated by UV radiation, containing phenol and merthiolate as preservatives and 5% glucose as stabilizer. This vaccine containing inactivated virus does not present specific rabies virus hazards. A potential immunopathological risk exists, however, due to the encephalitogenic factor related to a protein similar to mouse brain myelin [18,36,55,110], which has not been described in rats. Improvement of this production technique made it possible to avoid post-vaccinal accidents. This vaccine has been employed in Argentina since 1981 and only a few isolated cases of human rabies have been reported during the last 14 years.

Despite ethical considerations, the use of animals as a substrate for vaccine production remains of interest. Thus, recently, vaccines against aphthovirus have been proposed, employing radioactively immunosuppressed animals, with a high viral titer.

Second generation vaccines

Vaccines produced in cell cultures

In 1930, Stoel attempted to infect a primary chicken embryo brain culture for the first time [102]. During the following 10 years, propagation of fixed rabies virus strains in mouse embryo brain culture was reported [124]. In 1942, Plotz and Reagan infected a primary chicken embryo cell culture with 'street' rabies virus [80]. Tumoral cells have also been used for virus culture and isolation. In 1953, Sanders *et al* published the results of an assay on rabies virus adaptation to cell culture [88]. In 1956, Vieuchange described a non neural cell susceptible to rabies virus [119]. In 1958, Kissling [46] achieved the first significant advance, describing rabies virus multiplication in primary cultures of hamster kidney cells, employing a fixed CVS virus strain and obtaining titers of $10^{3.5}$ LD_{50} in mice. It was not until 1960 that Fenje [27] obtained the first rabies vaccine produced in cell culture, employing a SAD virus strain originally isolated from a rabid dog brain. Since then, researchers have originated several vaccines in primary cell cultures of different types. Currently, cell culture vaccines may be classified in three groups depending on the cell system employed for their production:

First group: The first group comprises vaccines manufactured in primary mammalian cell cultures, such as hamster kidney [28,63–65,92–94], dog kidney [113–115] and fetal calf kidney [4–6,35,103], or in avian cell cultures, such as chicken embryo [11,15,23,49,67,89,96,122] and quail embryo [13], following Kissling's methodology.

Second group: Second group vaccines are produced in diploid cells of regular cariogenicity and duplication, mainly of human origin (WI38 [9,126,127], MRC5 [41,84,128]). Some vaccines of animal origin (*Rhesus* monkey fetus [14,17]) likewise pertain to this group.

Third group: The third group includes vaccines developed in heteroploid cell culture as the Vero line [24,31,47,72,105].

The development of rabies vaccines for veterinary use, which are usually employed for prevention (one annual dose for dogs), was more widely favored than that of vaccines for human use. This preference is due not only to a greater demand for the former but also because production requirements are less stringent for veterinary vaccines. The main features of production processes for these rabies vaccines are listed in Table 2. Thus, for instance:

- BHK21 [25,56–61,68,77,83,109,125] and NIL2 [21,82] cell lines, where rabies virus replicates with very good yields reaching a high viral titer (10^6–10^7 LD_{50} in mice), have been accepted as substrate. These cell types allow an easy industrial scale up, performing culture in different systems (roller, spinner flask, stirred bioreactors, airlift and hollow fiber), thus increasing the yields.
- Vaccines manufactured with live attenuated virus are permitted for animal immunization [1–3] allowing oral vaccination of wildlife, employing rabies virus strain SAD [100,101] or SAD-B19 [16,19,30] clone.
- Addition of certain adjuvants is permitted, enhancing vaccine action [28,51,78].

Vaccines for human use are usually employed for treatment after exposure to rabies virus (five or more doses, depending on the type of vaccine) and have much more stringent requirements, which pose a challenge for their development, such as:

- Vaccines must be fully inactivated.
- Normal diploid cariotype cells employed as substrate for rabies virus replication must contain neither oncogenes nor be transformed.

Many studies and extensive investigation carried out to test potential oncogenic damage in continuous cell lines have disclosed that some of them, such as the Vero line (derived from the African green monkey *Cercopithecus Aethiops*), are free of these oncogenic properties and present no risks for human health when used in vaccine manufacture [48,71,120].

The main features of production processes for these rabies vaccines are listed in Table 3. Steps followed in their manufacture are as follows:

(1) Cell culture and amplification: Conventional culture in Roux or roller bottles is one of the amplification systems employed. In order to scale up and increase the yield, spinner flasks (Figure 1) and bioreactors (Figure 2) are used [31,70,72]. Among the different types of reactors, those with magnetic stirring, airlift and hollow fiber are the most widely used. Both airlift and hollow fiber reactors afford the advantage that they generate low shear forces to which cells are very sensitive [87].

An excellent alternative to obtain high cell concentrations when working with anchoring dependent cells is the use of gelatin microsupports (microcarriers) [73,116–118] (Figure 3). The greater surface/volume relationship present in this system is a major advantage, as it allows a

Table 2 Main features of rabies vaccines for veterinary use produced in cell cultures

Cell substrate	Virus strain	Vaccine type	Inactivation method	Use	Route	Reference
HK	CVS	Inactivated	βPL	Dogs, cats, cattle, sheep, goats, horses	I	Fromm Laboratories
NIL 2	PV	Inactivated	βPL	Dogs, cats, cattle	I	[82]
BHK	PV	Inactivated	EI, AEI, BEI, βPL	Dogs, cats, cattle, sheep, goats, horses	I	[56–58]
HK	Flury LEP	Attenuated	–	Dogs	I	Fromm Laboratories
CEF	Flury LEP	Attenuated	–	Dogs	I	Lederle Laboratories
DK	Flury LEP	Attenuated	–	Dogs, cattle	I	Norden Laboratories
PK	ERA	Attenuated	–	Dogs, cats, cattle, sheep, goats, horses	I	Connaught Laboratories
BHK	ERA	Attenuated	–	Dogs, cats, cattle, sheep, goats, horses	I	[1,2]
HK	Vnukovo-32	Attenuated	–	Dogs, cats, cattle, sheep, goats, horses	I	[93,94]
HK	SAD	Attenuated	–	Foxes (wild animals)	0	[100,101]
BHK	SAD-19	Attenuated	–	Foxes (wild animals)	0	[16,19,30]
BHK	SAG1	Attenuated	–	Foxes (wild animals)	0	VIRBAC France

HK: Hamster kidney; BHK: baby hamster kidney; CEF: chick embryo fibroblast; DK: dog kidney; PK: pig kidney; βPL: β-propiolactone; EI: ethylenimine; AEI: acetylenimine; BEI: binary-ethylenimine; I: injectable; O: oral.

Table 3 Rabies vaccines for human use produced in cell cultures: main features

Cell substrate	Virus strain	Concentration method	Inactivation method	Adjuvant	Pre-exposure treatment	Post-exposure treatment
WI 38	PM 1503	UF	TNBP/Tween-80	–	0, 7, 21, 28	0, 3, 7, 14, 30, 90
MRC-5	PM-1503	UF	βPL	–	0, 28	0, 3, 7, 14, 30, 90
MRC-5	PM-1503	ZC	βPL	–	0, 30, 60	0, 3, 7, 14, 30, 90
MRC-5	SAD-60	UF	βPL	–	E	E
VERO	PM-1503	UF-ZC	βPL	–	0, 7, 28	0, 3, 7, 14, 30, 90
HKC	Vnukovo-32	C	UV	–	0, 3, 7, 28	0, 3, 7, 14, 30, 90
HKC	Beijing-31	C	Formol/Phenol	$Al(OH)_3$	0, 3, 7, 28	0, 3, 7, 14, 30, 90
DKC	PM-1503	UF-Sepharose 6B	βPL	$AlPO_4$	0, 28, 180	0, 3, 5, 7, 14, 30, 90
FBKC	PV11/RV31	UF-ZC	βPL	–	0, 21	E
PCEC	Flury HEP	UF-C	βPL or UV	–	0, 28, 180	0, 2, 4, 6, 8, 14, 28
PCEC	Flury LEP	UF-C	βPL	–	0, 28, 180	0, 2, 4, 6, 8, 14, 28

Day of administration, q.d.
HKC: hamster kidney cell primary culture; DKC: dog kidney cell primary culture; FBKC: bovine foetus cell primary culture; PCEC: chick embryo cell primary culture; UF: ultrafiltration; ZC: zonal centrifugation; C: centrifugation; TNBP: Tri(*n*)-butyl-phosphate; βPL: beta-propiolactone; UV: ultraviolet radiation; E: Experimental.
Sources: [54,85].

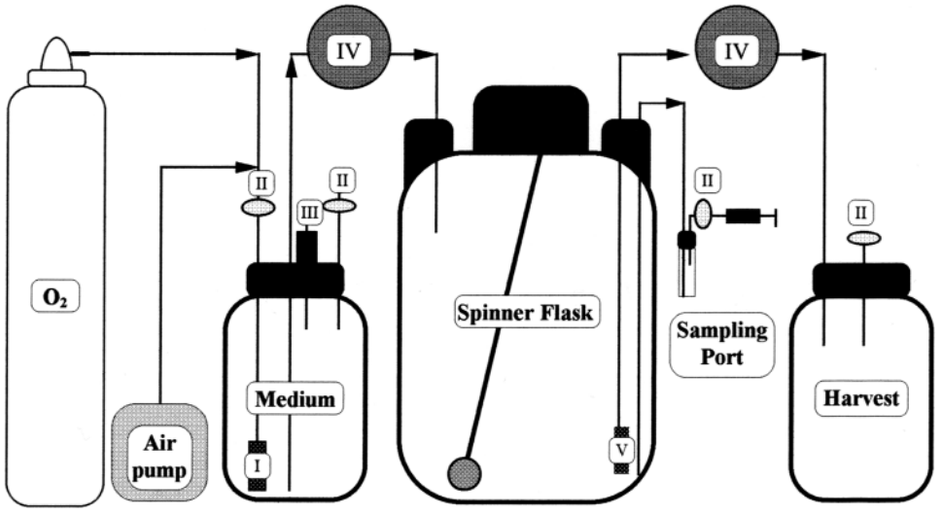

Figure 1 Configuration of the spinner flask perfused system employed for cell cultures on microcarriers. I: Air sparger; II: air filter (0.22 μm); III: $NaHCO_3$; IV: peristaltic pump; V: microcarriers filter (100 μm).

large number of cells to be obtained with a great reduction in work units. Thus, cell density reached in BHK21-C13 and Vero cell cultures on microcarriers is roughly 2×10^6 cells ml^{-1} using spinner flasks with medium rechanging systems. In perfused bioreactors, cell density with these cell lines reaches 2×10^7 cells ml^{-1} when the process is optimized by measurement and adjustment of variables such as pH, rate and type of stirring, percentage of dissolved oxygen, nutrient concentration (mainly glucose and glutamine), toxic metabolite concentration (mainly ammonium and lactate) and temperature. Bioreactor yield expressed as a function of the medium employed is 4- to 5-fold higher than in a conventional roller system and 1.4-fold higher than in spinner flasks. Evaluation of kinetic parameters for nutrient consumption and toxic metabolite production, as well as their relationship with cell growth, allows the yield to be enhanced by correlating an adequate number of cells to maximum antigen production.

(2) Infection and viral multiplication: Cell infection may be carried out in a suspension or in a monolayer. Virus adsorption may be favored in some cases by adding certain polyions [44] such as DEAE-DEXTRAN. In order to reach a maximum yield in viral productivity some intrinsic factors related to the cell-virus couple (including incubation temperature, infection multiplicity, time and harvest number), and some extrinsic factors related to the employed methodology must be taken into account. Correlation between cell density and antigen concentration achieved after infection is a complex function. In theory, once infection conditions are optimized, the greater the number of cells, the larger the number of produced virus. However, this is not always the case, probably due to the fact that there is no homogeneity in the metabolic status of all cells. Mass and energy transfer processes in systems with high cell density are complex and closely related to metabolic functionality.

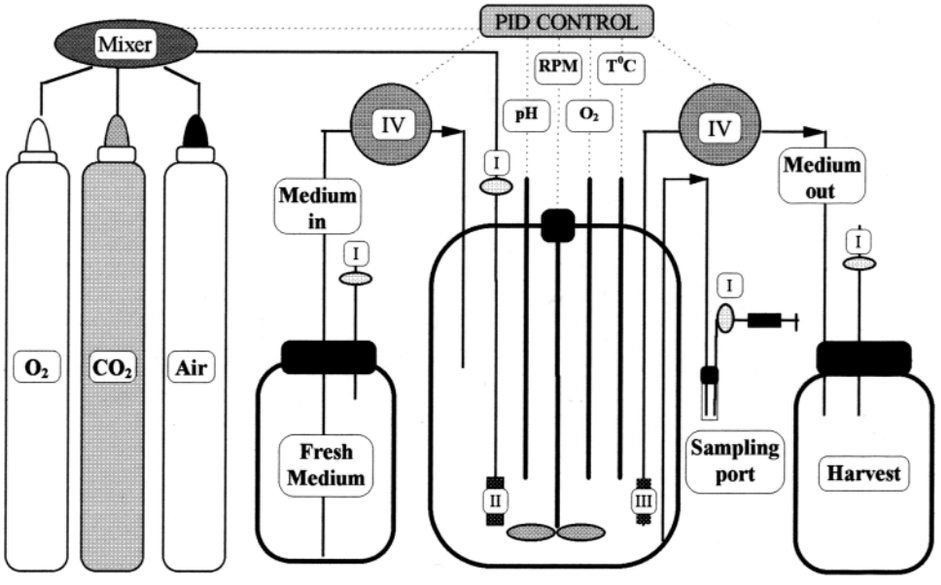

Figure 2 Configuration of the bioreactor with magnetic stirring employed for cell cultures on microcarriers. I: Air filter (0.22 μm); II: air sparger; III: microcarriers filter (100 μm); IV: peristaltic pump; PID Control: proportional integrative derivative control.

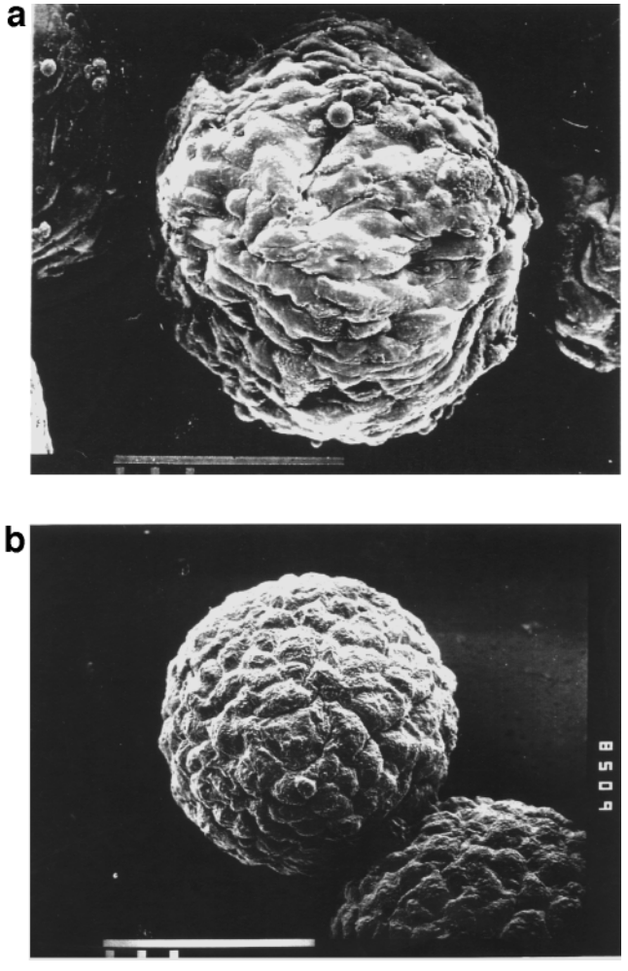

Figure 3 Scanning electron micrographs: confluent monolayers of (a) BHK21-C13 cells on gelatine non-porous microcarriers and (b) Vero cells on gelatine non-porous microcarriers. White bar = 100 μm.

For example, virus production in Vero cells cultured in a roller system, and infected with a PV-RV31 strain (derived from Pasteur virus PV), is carried out employing a 33°C incubation temperature, infecting in suspension as described by Coonoor *et al* [111], with an infection multiplicity of 0.2 LD_{50} $cell^{-1}$, and carrying out 3–4 harvests every 3 or 4 days.

(3) Concentration and purification: For vaccines produced in cell culture, viral antigen concentration and purification processes are very common. Many methods have been described for this purpose [12], some of which are shown in Table 3. In large-scale production, the most commonly employed techniques are ultrafiltration and centrifugation in a continuous density gradient. The first technique is a simple and efficient method to concentrate antigen but its main disadvantage lies in the fact that several undesirable proteins are also concentrated. Although the second technique is more complex, it is excellent not only to concentrate but also to purify materials.

(4) Inactivation: Inactivation is mandatory for vaccines to be used in humans. Several methods are available for rabies virus inactivation (see Table 3). If a chemical-inactivating reagent is used, concentration, inactivation temperature and time must be established for each type of vaccine. One of the most successfully employed agents for these types of vaccines is β-propiolactone used in a heated environment. This method has proven to be the best method for inactivating rabies virus leaving the lowest amount of residual DNA from the cell substrate [74]. Its main disadvantage lies in the high cost of the reagent, and its extreme toxicity before thermal treatment. Ultraviolet radiation is also employed, in which case, dosage and application times are critical in order to achieve total virus inactivation without diminishing antigenicity.

(5) Stabilization and lyophilization: Most of these vaccines are available in a lyophilized form, which allows

higher stability. Due to this, the expiration date is almost a year longer than that for material produced in liquid form.

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