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PROPERTIES OF RABIES STRAIN („PASTEUR POTSDAM") ADAPTED TO PRIMARY DOG KIDNEY CELLS

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Summary. — The Pasteur strain of fixed rabies virus (Pasteur Institute Paris, passage 2061 in rabbit brain) was adapted by alternate passages to primary dog kidney cells. The adapted rabies virus designated as "Pasteur Potsdam" developed no CPE and yielded four harvests with a titre of 5.5—7.0 (log $MICLD_{50}$/ml). The strain could be grown in BHK 21/S13, CER and N2a neuroblastoma cells. In the cultures of BHK 21/S13 cells the virus titered 6.0—8.5 (log $MICLD_{50}$/ml). In SDS PAGE its G protein migrated faster than that of the ERA strain. The inactivated antigen induced interferon in mice. The strain was identified by anti-rabies immunoglobulin. The harvested material showed an antigenic value of 0.4 IU/ml. The virus was not pathogenic after s.c. and i.p. inoculations to mice, rats, Syrian hamsters, and rabbits and after i.m. inoculation to Syrian hamsters and rats.

Key words: *rabies virus*; *adaption*; *cell cultures*

Introduction

For many years rabies strains have been maintained by intracerebral (i.c.) inoculation of mammals or by multiplication in chicken or duck embryos. The rabies vaccines from brains caused a large number of neurological complications. Vaccines from virus grown in cell cultures contain less neuroallergenic substances. The first successful cultivation of rabies virus in cell culture was reported by Kissling (1958). The adaption of the Pitman-Moore strain to the human WI 38 diploid cell was a very important step to a new generation of rabies vaccine (Wiktor *et al.*, 1964). Another cell culture rabies vaccine was produced in primary Syrian hamster kidney cells infected by Vnukovo 32, an original SAD strain (Selimov *et al.*, 1978). The chicken embryo cell-adapted Flury-strains are also used (Kondo *et al.*, 1978; Barth *et al.*, 1984) for rabies vaccine. In addition, the original Pasteur strain was adapted to primary bovine foetal kidney cells (Atanasiu *et al.*, 1974) and to Japanese quail embryo cells (Bektemirova *et al.*, 1979).

We describe the adaption of an original Pasteur fixed virus to primary dog kidney cells and the properties of this strain. The strain may be used as a human vaccine candidate.

Materials and Methods

Cell cultures. Dog kidney cell cultures were prepared by trypsination of 2—4 weeks old Beagle dogs (closed colony). The cells were grown in a medium containing Hank's solution and 10% calf serum. The cell concentration was 100 000 to 200 000 cells/ml. The monolayer was confluent after 7 days. BHK-21/S13 cells were cultured in Eagle's MEM, Glasgow modification, with 10% calf serum, the murine neuroblastoma cells N2a and the L 929 cells in Eagle's MEM with 10% calf serum.

Viruses. The Pasteur strain fix of rabies virus was supplied by the Pasteur Institute, Paris. The Pasteur strain was in rabbit brain passage no. 2061. First the strain had been adapted by alternate passages to baby mice and to cells. The brain material was adsorbed on the cells for 1 hr at 37 °C. The virus was grown at 35 °C in Hanks-gelatine-dextran-medium (0.25% gelatine, 0.6% dextran, 0.5% lactalbumin hydrolysate, pH 7.8—8.0) which contained 2% calf serum. After 3 days the medium was replaced by gelatine-dextran-medium without serum. Multiple harvests of virus suspension were obtained at intervals of 3—4 days.

The Pasteur Potsdam strain was grown in the BHK-21/S13, N2a, and CER cells in the presence of bovine serum albumin (0.1%) and without serum at 35 °C. The rabies strain ERA was cultured in the same way as the Pasteur Potsdam strain in the BHK-21/S13 cells. The multiplication of the vesicular stomatitis virus (VSV), Indiana strain, was made in L-929 cells.

Virus titration. The infectivity of the virus was determined in subadult (10—14g) ICR mice (tenfold dilution). The animals were observed for 14 days.

Virus inactivation. The rabies virus was inactivated soon after the harvest by 0.025% beta-propiolactone for 18 hr at 4 °C.

Virus concentration and purification. PEG 6000 was added to the virus suspension in a final concentration of 6% (Mikhalovsky, 1971). The virus suspension was concentrated by ultracentrifugation.

Determination of protein. The content of virus protein was determined by the method of Lowry (Lowry *et al.*, 1967) in comparison with bovine serum albumin.

Polyacrylamide gel electrophoresis (SDS-PAGE). The method of Maizel (1967) was used in a modified way. Both 50 μg purified virus suspension of the strain ERA and Pasteur Potsdam were cleaved by 1% SDS, 2% mercaptoethanol by heating in a boiling water bath for 3 min. A 8% polyacrylamide gel was used as the running gel, a 5% gel as stacking gel. The material was fixed in acetic methanol and stained with Coomassie blue.

Interferon (IFN) assay. Each mouse received i.p. inactivated virus equivalent 1/10 of the usually recommended human dose (antigenic value > 2.5). By 3, 6, 9, and 24 hr post-vaccination 6 mice were bled and sera collected. The IFN assay was performed in a microtest. After incubation with mouse sera 50—100 $TCID_{50}$ of the VSV was given to the monolayer of L-929 cells; the results were read 24 hr later. The laboratory mouse IFN was compared with the International Reference Mouse Interferon Standard (G002-904-511).

Neutralization tests. Neutralization of the virus was made at decreasing virus concentrations with a constant serum dilution (1 or 7 IU/ml human immunoglobulin anti-rabies) or at constant virus concentration and with decreasing serum dilutions in 10—14g ICR mice observed for 14 days.

Investigation of antigenicity, immunogenicity, and virulence. The antigenic activity of virus strains was determined by i.m. and s.c. immunizations of mice (10—14g) and guinea pigs (250—300g) with inactivated virus suspension (3 times weekly). The animals were bled 7 and 14 days after the last dosis. Antibody titres in blood serum were determined by a neutralization test in mice. The immunogenicity of the inactivated virus antigen was ascertained by the method of the National Institute of Health (NIH), U.S.A. (Seligman, 1973).

During the adaption of the Pasteur strain the intracerebral and extraneural (i.m., s.c., i.p.) virulence, the length of incubation period and of survival time were investigated in mice (NMRI 10—14g), rats (Wistar 80—120g), Syrian hamsters (50—60g), and rabbits (2.5—3kg). The specificity of death cases was verified by the indirect fluorescence antibody test.

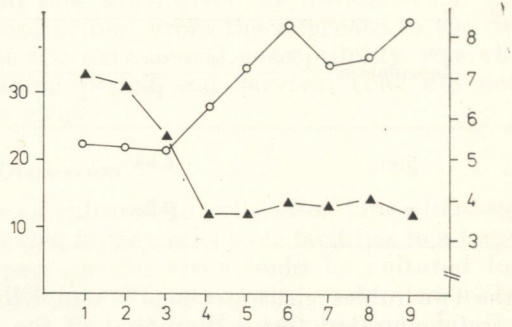

Fig. 1.
Course of the harvest with the highest titre in dependence of the number of cell passages
● virus titre (log $MICLD_{50}$/ml, ▲ duration (days after infection).
Abscissa: cell passages; ordinates: in the left — duration (days), in the right — virus titre (log $MICLD_{50}$/ml).

Results

After 4 alternate passages in baby mouse and dog kidney cells virus multiplication in cells became possible. The adaption could be accelerated if the virus suspension for next cell passage was harvested (at intervals of 3—4 days) by 7—14 days after the titre culmination. During adaption, the duration of the harvest with the highest titre (optimal harvest) decreased with increasing cell passages (Fig. 1).

In the first cell passages the optimal harvest was obtained 20—30 days p.i. From the 5th cell passage the optimal harvest was reached already after 10—14 days p.i. The rabies strain adapted to primary dog kidney cells was called Pasteur Potsdam (PP). The strain yielded in dog kidney cells four harvests with the infectious titre of 5.5—7.0 (log $MICLD_{50}$/ml).

The PP strain could also be cultured in continuous cells. The virus suspension which was taken from the dog kidney cells was incubated with the same titre on BHK-21/S13 cells, CER cells and neuroblastoma cells and passaged after that. The PP strain reached in the 3 continuous cell lines constant titres already after the 2nd passage. In cultures of BHK-21/S13 cells the titre of the virus was 6.0—8.5, in CER cells 5.0—6.0 and in neuroblastoma cells 5.0—5.4 (log $MICLD_{50}$/ml), respectively.

The SDS PAGE showed no differences between the nucleocapsid protein (N), the core protein (NS) and the membrane protein (M) in comparison to

Table 1. Interferon inducing capacity of the rabies strain Pasteur Potsdam in mice in dependence on time

Time (hours after i.p. vaccination)	Interferon titres (IU)
3	4
6	16
9	<4
24	<4

Table 2. Antibody induction of the Pasteur Potsdam strain in guinea pigs (1 injection on days 0.7 and 14) in titres (IU/ml) on day 21

Inoculation	Cell passage		
	17	18	20
i.m.	6.88	30.63	237.50
s.c.	9.38	21.88	24.38

the two rabies virus strains PP and ERA. The glycoprotein (G) of the PP strain migrated faster than that of the ERA strain. The large protein (L) could not be always identified. The interferon inducing capacity of the PP strain is documented in Table 1. Six hours after i.p. vaccination the highest interferon titres were reached. In the neutralization test the PP strain showed neutralization indices about 300 at constant serum dilution. Using 10—30 $MICLD_{50}$ of virus serum concentration between 1 : 20 and 1 : 45 (0.05—0.022 IU/ml) was necessary for the neutralization. The investigation of the antigenicity of the adapted virus strain showed that the guinea pig gave good antibody responses (Table 2). In comparison with the fixed rabies virus strains Pitman Moore, Novi Sad and Pasteur Paris the PP strain induced the highest antibody titres (>150 IU/ml) in mice and guinea pigs (virus dosis 316 LD_{50}).

The immunogenicity of the inactivated virus suspension between the 20th and 25th cell passage was 0.16 IU/ml—1.79 IU/ml with a geometric mean antigenic value of 0.4 IU/ml. The investigation of the PP strain virulence in mice, rats, Syrian hamsters, and rabbits demonstrated that in the course of adaption the intracerebral and in particular the extraneural susceptibility decreased (Table 3). Especially the subcutaneous and intraperitoneal infection of the adapted virus in adult mice, rats, Syrian hamsters, and rab

Table 3. Virulence of the Pasteur Potsdam strain in different passages

Inoculation	Mouse			Rat			Syrian hamster			Rabbit		
	BP1	CP12	CP22	BP1	CP12	CP22	BP1	CP12	CP22	BP1	CP12	CP22
i.c.	6.0	4.2	4.1	3.8	3.5	3.4	5.5	4.0	2.3	6.5	4.3	3.5
i.m.	3.4	2.1	1.7	3.5	0.5	0	4.5	2.2	0	1.0	0.5	0.5
s.c.	2.0	0.5	0	1.5	0.5	0	2.3	1.6	0	1.0	0.5	0
i.p.	2.5	0.5	0	2.2	0.5	0	3.5	2.0	0	0.5	0.5	0

BP — brain passage of the original Pasteur rabies strain; CP — cell passage; titre: LD_{50}; doses: mouse: i.c. — 0.03 ml, i.m. — 0.5 ml, s.c. — 1.0 ml, i.p. — 1.0 ml; rat: i.c. — 0.1 ml, i.m. — 2.5 ml, s.c. — 2.5 ml, i.p. — 3.0 ml; Syrian hamster: i.c. — 0.05 ml, i.m. — 1.0 ml, s.c. — 1.5 ml, i.p. — 2.0 ml; rabbit: i.c. — 0.25 ml, i.m. — 5 ml, s.c. — 7 ml, i.p. — 10 ml.

bits was not accompanied by any symptoms of disease. The strain was not pathogenic for Syrian hamsters and rats when given by intramuscular inoculation. Baby mice were more susceptible. From the alternate to the direct passage a distinct decrease of the extraneural susceptibility was observed in all animals. The incubation period and survival time did not change during the adaption of the virus.

Discussion

The fixed virus strain Pasteur was cultured in cells after four alternate passages. The good durability of the dog kidney cells gave facilities to a large number of harvests. During one passage the virus could be cultured for a long time in these cells. The adaption was particularly accelerated when the virus suspension was taken 7—14 days after the culmination of virus titre. In the course of the adaption the optimal harvest appeared within a shorter period. This can be considered as a marker of adaption. An advantage of the primary dog kidney cell culture is the possibility of obtaining four virus harvests with an infectious titre of 5.5—7.0 (log $MICLD_{50}$/ml). This titre is quite comparable with titres of other rabies virus strains (Wiktor *et al.*, 1964; Selimov *et al.*, 1978; van Wezel *et al.*, 1978; Bektemirova *et al.*, 1979; Barth *et al.*, 1984; Montagnon *et al.*, 1985).

The PP strain could be grown in BHK-21/S13 cells, CER and neuroblastoma cells as well. This may be regarded for a marker of good adaption. The titres of virus strain in BHK-21/S13 cells were 6.0—8.5 (log $MICLD_{50}$/ml). The PP strain prepared in this continuous cell line could be used as veterinarian vaccine.

The PAGE showed differences in the position of the glycoprotein G of PP and ERA rabies strains. In accordance with Arita and Atanasiu (1980) the glycoprotein of the PP strain migrated faster than that of the ERA strain. The interferon inducing capacity of the PP strain in mice was pointed out. The titres of interferon are of the same size as stated by other authors (Fornosi *et al.*, 1985). The identity of the PP strain as an rabies virus could be confirmed by neutralization with rabies serum and by NIH test.

The PP strain induced satisfactory antibody levels. This was observed in guinea pigs. The titres were higher than in the 3 other fixed rabies virus strains. The determination of the immunogenicity of the adapted strain gave a mean antigenic values of 0.4 IU/ml. As a result of the obtained antigenicity and immunogenicity we suppose that the PP strain is suitable for human rabies vaccine production. Subcutaneous and i.p. inoculations of the virus into four tested animal species were not accompanied by any clinical symptoms of disease. After i.m. inoculation the Syrian hamsters and rats were not susceptible to the strain. The PP strain corresponds in these properties with the other strains used for rabies vaccination (Wiktor *et al.*, 1964; Majdan *et al.*, 1974; Rulka *et al.*, 1974; Aksenova, 1976; Bektemirova *et al.*, 1979).

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References

Aksenova, T. A. (1976): The biological, immunological, and morphological characteristics of fixed rabies virus (SAD) strain used in the production of tissue-culture rabies vaccine, pp. 74—76. In *Documentation of the Thirteenth Session of the Institute of Poliomyelitis and Viral Encephalitis of the Academy of Medical Science of the U.S.S.R.* Moscow.

Arita, M., and Atanasiu, P. (1980): Comparative study of the molecular weight of the structural protein from rabies using polyacrylamide gel electrophoresis. *Ann. Virol. (Inst. Pasteur)* **131E**, 201—215.

Atanasiu, P., Tsiang, H., and Gamet, A. (1974): Nouveau vaccin antirabique humain de culture cellulaire primaire. *Ann. Microbiol. (Inst. Pasteur)* **125***B*, 419—432.

Barth, R., Gruschkau, H., Bijok, U., and Hilfenhaus, J. (1984): A new inactivated tissue culture. rabies vaccine for use in man. Evaluation of PCEC-vaccine by laboratory tests. *J. biol. Stand.* **12**, 29—46.

Bektemirova, M. S., Osidze, D. F., Phille, E. R., Nadaichik, L. V. (1979): Properties of rabies virus (MNIIVP-74 strain) adapted to Japanese quail embryo cell culture. *Arch. virol.* **61**, 61—68.

Fornosi, F., Thales, M., and Solomo, G. (1985): Studies on the interferogenicity and immunogenicity of different types of rabies vaccines, pp. 205—208. In E. Kuwert, C. Merieux, H. Koprowski (Eds.): *Rabies in the Tropics*, Springer Verl. Berlin.

Kissling, R. E. (1958): Growth of rabies virus in nonnervous tissue culture. *Proc. Soc. exp. Biol. Med.* **98**, 223—225.

Kondo, A. (1978): Preimmunization and post exposure treatment with inactivated rabies vaccine of chick embryo cell origin. *Develop. biol. Stand.* **40**, 147—153.

Lowry, O. H., Rosenbrough, N. J., Farr, L., and Randell, R. J. (1951): Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265—275.

Maizel, J. V. Jr. (1971): Polyacrylamide gel electrophoresis of viral proteins. *Meth. virol.* **5**, 180—246.

Majdan, St., Rulka, J., and Nikiel, J. (1974): Adaption of Flury HEP strain of rabies to Syrian hamster kidney cell culture. *Acta microbiol. Polon.* **6**, 113—116.

Mikhajlowski, E. M. (1971): Concentration du virus rabique par le polethylene glycol. *Ann. Inst. Pasteur* (Paris) **121E**, 563—568.

Montagnon, B. J., Fournier, P., and Vincent-Falquet, J. C. (1985): Un nouveau vaccin antirabique a usage humain: rapport preliminaire, pp. 138—143. In E. Kuwert, C. Merieux, H. Koprowski (Eds): *Rabies in the Tropics*, Springer Verl. Berlin.

Rulka, J., Majdan, St., and Nikiel, J. (1974): Adaption of rabies virus (Flury LEP strain) to hamster kidney cell culture. *Acta microbiol. Polon.* **A6**, 118—122.

Seligman, E. B. Jr. (1973): The NIH test for potency. *WHO Lab. Techn. in Rabies* (3rd ed) **8**, 279—286.

Selimov, M., Aksenova, T., Klyneva, E., Gribencha, L., and Lebedeva, I. (1978): Evaluation of the inactivated tissue culture rabies vaccine from the Vnukovo-32 strain. *Develop. Biol. Stand.* **40**, 57—62.

Van Wezel, A. L., and van Steenis, G. (1978): Production of an inactivated rabies vaccine in primary dog kidney cells. *Develop. biol. Stand.* **40**, 69—75.

Wiktor, F. J., Fernandes, M. V., and Koprowski, H. (1984): Cultivation of rabies virus in human diploid cell strain WI-38. *J. Immunol.* **93**, 353—366.