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MULTIPLICATION AND DISTRIBUTION OF ATTENUATED PSEUDORABIES VIRUS IN THE ORGANISM OF VACCINATED PIGS

O. JAMRICHOVÁ, R. ŠKODA

Institute of Virology, Czechoslovak Academy of Sciences, Bratislava

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Summary. — After subcutaneous (sc) injection of both low (10^2 PFU) or high (10^6 PFU) doses of the vaccine strain BUK of pseudorabies virus (PRV) into the flank of pigs, the virus was regularly detected 2–8 days post infection (p.i.) in the subcutaneous tissue at the site of inoculation (SI), in the regional inguinal lymph nodes (ILN), and in 18% (6 of 33) of vaccinated pigs also in the central nervous system (CNS) (brain and lumbal spinal cord). Various other organs, body fluids and excrements were found free of virus (only one out of 250 blood samples tested was positive). A similar distribution was also observed with the vaccine strain TK 200 which, however, was detected in the suprarenal gland of 1 out of 3 piglets infected sc into the flank. The use of low virus doses provided evidence that PRV actively multiplies at the SI, even though to low titres only. It cannot be definitely concluded whether PRV actually multiplied in lymph nodes (LN) or whether it was just transported there after it had multiplied at the SI, especially since the virus titres in LN were frequently low as compared to those in primarily inoculated tissues.

Introduction

Although a number of various live vaccines prepared from modified PRV have been used for a long time in the prevention of Aujeszky's disease in pigs and other domestic animals in the countries of central and south-east Europe, only the Rumanian authors of the avianized vaccine (Bran *et al.*, 1963) reported some data on the distribution of the vaccine virus in the organism of immunized pigs. However, informations of this kind are important not only for the evaluation and comparison of different vaccines, but also for understanding of the mechanisms of the immunological response.

Materials and Methods

Viruses. The stock suspension of the modified strain BUK in its 622nd passage in chick embryo cells (CEC), prepared as described before (Škoda *et al.*, 1964), was stored at 4° C. When used it had a titre of at least 10^6 plaque forming units (PFU) per ml. The TK 200 virus (Žuffa, 1963*a*) was the commercial product of Bioveta, Nitra, namely the liquid vaccine lot No. 37 with titre of 2×10^6 PFU/ml. The virulent virus ČVOŠ, isolated from a pig with naturally acquired Aujeszky's

disease, was used in its 7th CEC passage and had a titre of 6×10^5 PFU/ml. In order to mark precisely the SI, finely ground charcoal (about 3%) was usually added to the given virus dose just before inoculation.

Cell cultures. The preparation of tube and plate cultures of CEC was described previously (Škoda *et al.*, 1964). Virus titrations and isolation experiments were carried out in parallel in tube and plate cultures. Neutralization tests were done in tube cultures.

Pigs. Weanlings (7—8 weeks old) and 6 days old sucking pigs were supplied from a breed free of Aujeszky's disease; they had no neutralization antibody ($1 : <2$) when entering the experiments. Altogether 56 pigs were used in 6 experiments. The given virus dose in 1 ml was inoculated sc (about 5 cm aside the pierced skin) close to the flank on the inner side of the thigh. The animals were killed at intervals; they were anaesthetized by intravenous administration of thiopental and exsanguinated from the vena cava cranialis, using a large diameter cannule.

Specimens. In the isolation experiments from pigs infected with modified viruses BUK or TK 200, the following organs and fluids were tested; olphactory bulb, cerebral cortex from the parietal lobe and the basal part of frontal lobe of the hemispheres, cerebellum, brain stem, nasal mucosa, tonsils, tracheal mucosa, lungs, parotis and submandibular salivary glands, liver, spleen, kidneys, suprarenal glands, subcutaneous tissue at the SI, inguinal and popliteal LN, femoral and diaphragmatic muscles, mucosa of the small and large intestine, blood, bile and urine. In the pigs infected with virulent virus ČVOŠ, isolation experiments were performed only with organs indicated in Table 1.

Solid specimens were ground in mortar with sterile sea sand and the medium for CEC culture, containing an increased concentration of antibiotics (1000 units/ml penicillin and 1000 μg/ml streptomycin; occasionally also 100 units/ml of mycostatin), to make 10% suspensions. After clarification by centrifuging, tenfold dilutions in the medium mentioned were prepared from the materials and inoculated into tube cultures (0.1 ml aliquots in quadruplicates) and plate cultures (0.4 ml aliquots in duplicates). In the case of plates, the inoculum was removed after 90 minutes of adsorption and the agar overlay with neutral red (1 : 30 000) was add d. Tube cultures were freed of inoculum only in the case of low dilutions of suspensions prepared from the liver, spleen, LN, mucosa, blood, bile and urine. Since of the viruses regularly occurring in pigs only PRV multiplies in CEC, we considered the appearance of a characteristic cytopathic effect (CPE) (final readings after 5 days) or of plaques (after 4—5 days) as a sufficient criterion of the presence of PRV. Neutralization tests were not employed to identify the virus. Virus was passaged suspect plaques (especially when only 1—2 plaques in one plate had appeared) in tube CEC cultures or in rabbits (sc inoculation into the radix auriculae).

Blood from weanlings was tested for virus in 3 experiments; blood samples were taken daily from v. cava cranialis and inoculated into CEC tube cultures.

Neutralization tests. Equal volumes of twofold serum dilutions and 100—500 $TCID_{50}$ of BUK virus were mixed, incubated at 37° C for 1 hour and 0.2 ml portions inoculated into quadruplicate CEC tube cultures. As titre of neutralizing antibody was taken that dilution of serum which inhibited the CPE in half of the inoculated tubes.

Results

Multiplication of virulent PRV in weanlings after sc infection

With regard to a rather large number of reports on the isolation of PRV from organs of pigs experimentally infected with virulent strains (Sabó *et al.*, 1968*a*, *b*; Rajčáni *et al.*, 1969), we carried out the control experiments on sc infection with the virulent virus using only 4 piglets (Table 1).

In one animal killed 2 days p.i., showing no clinical signs, PRV was detected at the SI and in the corresponding ILN. The other 3 animals fell ill clinically 3 days p.i. (all showed elevated temperature, weakness, aphony, loss of appetite). Two of them were killed 5 and 7 days p.i., respectively, and contained at the SI and in the ILN more virus per gram of tissue than was the amount of virus applied. In addition, PRV was also detected in tonsils, lungs, spleen and lumbal spinal cord. On the 7th day p.i., the tem-

Table 1. Multiplication of virulent PRV in weanlings after sc infection with 5×10^3 PFU

Pig No.	Days p. i.	Virus titre in PFU/g					
		SI	ILN	Tonsils	Lungs	Spleen	Lumbal spinal cord
1	2	$>5 \times 10^4$	$>5 \times 10^4$	—	—	—	—
2	5	$>5 \times 10^3$	$>5 \times 10^3$	1×10^2	5×10^3	—	—
3	7	$>5 \times 10^4$	$>5 \times 10^3$	—	3×10^2	3×10^2	5×10
4	10	—	—	—	—	—	—

Other organs examined (popliteal lymph node, liver, kidney, cerebral cortex, cerebellum and brain stem) and blood were negative.
— = No virus detected.

perature of the fourth animal returned to normal and it began to eat; it was killed on the 10th day p.i. Virus was not found in any of its organs tested, but its serum contained neutralizing antibody in a titre of 1 : 32.

The distribution of BUK virus in weanlings after sc inoculation of 10^6 and 10^4 PFU

In the first experiments, the weanlings were infected with the virus dose used for vaccination, i.e. 10^6 PFU (Table 2). None of the 19 infected animals

Table 2. Presence of the BUK virus in weanlings after sc inoculation of 10^6 and 10^4 PFU

Pig No.	Inoculum (PFU)	Iterval p. i.	Virus titre in PFU/g			
			SI	ILN	CNS	Blood
5		1 day	3×10^5	10^4	—	—
6		2 days	6×10^4	—	—	—
7		2 days	3×10^5	—	—	—
8		2 days	3×10^5	2×10^4	—	—
9		4 days	1.5×10^3	—	—	—
10		4 days	—	1.5×10^2	5×10^2	2×10
11		4 days	1.2×10^3	1.9×10^3	—	—
12	10^6	4 days	10^2	2×10^2	—	—
13		5 days	2×10^2	2×10	—	—
14		5 days	3×10^3	3×10^3	—	—
15		6 days	10^2	3×10	2×10^2	—
16, 17		6 days	—	—	—	—
18		7 days	—	2×10	—	—
19, 20		8 days	—	—	—	—
21, 22		10 days	—	—	—	—
23		24 days	—	—	—	—
24		1 hr	2.4×10^2	—	N	N
25	10^4	8 hr	3.4×10^3	2.5×10^2	—	N
26		2 days	10^3	10^2	2.5×10^3	—

Virus in CNS: in animals Nos 26 and 15 in cerebral cortex;
in animal No. 10 in cerebral cortex (10^2 PFU/g) and brain stem (5×10^2 PFU/g).
— = No virus detected. N = Not done.

fell ill with clinical symptoms. In 6 out of 7 animals killed 1, 2 and 4 days p.i., PRV was detected at the SI and/or the corresponding ILN, but the amount of virus per gram of tissue never exceeded or reached the dose of virus inoculated. In only one animal was a very low virus titre found 4 days p.i. in the CNS and even in blood (this was the only positive case out of 200 isolation experiments from 25 pigs). In animals killed 5, 6 and 7 days p.i., the virus content at the SI and in the ILN decreased gradually, so that after 8, 10 and 24 days p.i. PRV could not be detected at all. In 1 of these 5 animals, PRV was again found in CNS.

It was possible to conclude that even after a high infecting dose, the BUK virus regularly does not spread throughout the organism, except a relatively rare penetration of virus into the CNS and blood. Considering the virus

Table 3. Multiplication of the BUK virus in weanlings after se inoculation of 10^3 and 10^2 PFU

Pig No.	Inoculum (PFU)	Interval p.i.	Virus titre in PFU/g			
			SI	ILN	CNS	Blood
27	10^3	1 hr	—	—	N	N
28		1 day	2×10^3	2×10^2	—	N
29		2 days	3×10^3	3×10^2	—	—
30		4 days	—	—	—	—
31	10^2	1 hr	—	—	N	N
32		1 hr	—	—	N	N
33		1 day	5×10	—	—	N
34		2 days	2×10^2	—	—	—
35		2 days	1.6×10^3	10^3	—	—
36		2 days	10^3	—	—	—
37		2 days	5×10^2	2×10^2	—	—
38		4 days	—	—	—	—
39		4 days	—	10^2	2×10	—
40		6 days	—	—	—	—
41		6 days	10^2	2×10	2×10	—
42		8 days	—	—	—	—
43		8 days	5×10^2	—	—	—

Virus in CNS: in animals Nos 39 and 41 in mesencephalon and lumbal spinal cord, respectively.
Viraemia was followed in animals Nos 34—43 twice daily from infection till killing; altogether 80 isolation experiments were performed, all of which were negative.
— = No virus detected.
N = Not done.

titres estimated at the SI and expressed per gram of tissue, it was impossible to state that this virus was just a residuum of the inoculated dose in the subcutaneous tissues, especially if the thermal inactivation of virus in the organism was not taken into account. Similarly, when considering the virus titres in the corresponding LN, it could not be decided whether the virus was just passively transported there from the SI via lymphatic routes, or whether multiplication of virus had preceded there.

Since a high inoculation dose may obliterate a weak active virus multiplication, sc inoculation of 10^4 PFU was used; this led to results similar to those of the preceding experiments. Just a rather high virus titre in the CNS of piglet No. 26 would seem to suggest that not only a passive virus dissemination was involved, but that PRV actively multiplied in selective tissues.

Table 4. Presence of vaccine viruses BUK and TK 200 in sucking pigs 5 days after sc inoculation of 10^4—10^6 PFU

Pig No.	Virus	Inoculum (PFU)	Virus titre in PFU/g			
			SI	ILN	CNS	Suprarenal gland
44	BUK	10^6	3×10^5	3×10^5	—	—
45		10^5	10^4	10^4	—	—
46		10^4	10^4	—	—	—
47	TK 200	10^6	3×10^3	10^2	—	
48		10^5	10^4	3×10^3	—	
49		10^4	10^6	10^4	10^2	10^3

Virus in CNS: in animal No. 49 in the cerebellum.
— = No virus detected.

Multiplication of BUK virus in weanlings after sc inoculation of 10^3 and 10^4 PFU

In further experiments (Table 3), substantially lower virus doses were used for inoculation. In animals killed from 1 hour to 6 days p.i., PRV was detected in 9 of 17 animals, as a rule again only at the SI and in the ILN. All the virus titres established (with a possible exception in the case of animal No. 33) indicated a weak but significant virus multiplication at the SI and probably also in the ILN. One hour p.i. PRV could not be found at the SI in any of the 3 animals tested. At 4—8 days p.i., PRV was detected irregularly at the SI and in the ILN in 3 of 7 animals; in only one of them (No. 41) was the virus found also in the CNS. In total, the presence of PRV in the CNS was demonstrated in 2 of 17 animals.

The presence of the vaccine viruses BUK and TK 200 in sucking pigs after sc inoculation of 10^4—10^6 PFU

Since BUK virus was demonstrated in the CNS of some of the sc infected animals, four 6 days old sucking pigs were inoculated sc into the flank with 10^4—10^6 PFU of BUK or TK 200 virus. The animals were killed 5 days p.i., i.e. at a time at which PRV was the most frequently detected in the previous experiments (Table 4).

None of the sucking pigs showed clinical symptoms (body temperature was not measured). The examination of the SI and the regional LN revealed markedly stronger local reactions in all sucking pigs as compared to weanlings. Besides that, the suprarenal gland of animal No. 46 showed hypertrophy, hyperaemia and necrotic foci.

The isolation experiments demonstrated virus at the SI and in the ILN of all sucking pigs. Virus in the CNS was found only in one animal which was infected with TK 200 virus; in the same animal, also the suprarenal gland was positive for virus and displayed considerable alterations.

Table 5. Presence of the BUK virus in immune piglets after sc inoculation of 10^6 PFU

Pig No.	NA titre*	Days p.i.	Virus titre in PFU/g	
			SI	ILN
50	1 : 2	1	–	–
51	1 : 2	2	–	2.5×10^3
52	1 : 4	3	2.5×10^3	–

* Titre of neutralizing antibody on the day of revaccination.
– = No virus detected.

The presence of BUK virus in immune weanlings after sc inoculation of 10^6 PFU

Piglets Nos 50, 51 and 52 originated from an immunized breed and were first vaccinated with BUK virus when 6 weeks old; at that time they still had neutralizing antibodies in titres of 1 : 2—1 : 4 (Table 5). The sc vaccination into the radix auriculae did not lead to a rise of neutralizing antibody titres. Two weeks after the first dose the animals were given a second dose of 10^6 PFU of BUK virus sc into the flank; they were killed 1—3 days later. PRV was irregularly detected at the SI or in the ILN in 2 piglets only. These findings and the rather low virus titres found lead us to assume that in the animals with a certain level of virus neutralizing antibody the detection of PRV at the SI is irregular and that PRV disappears rapidly from the SI an the ILN.

Table 6. Neutralizing antibody formation in weanlings vaccinated sc with 10^2 PFU of virus

Pig. No.	Neutralizing antibody titre						
	Days after 1st inoculation				Days after 2nd inoculation		
	0	36	57	75	8	35	70
53	0	0	0	0	0	0	0
54	0	0	0	0	32	128	16
55	0	6	4	4	64	128	128
56	0	0	0	0	0	0	0

0 means <2.

Formation of neutralizing antibody in weanlings after sc inoculation of 10^2 PFU of BUK virus

With respect to a significant but low multiplication of PRV at the SI after sc administration of a very low dose of virus (10^2 PFU, Table 6) we were interested in the immune response of the organism under such conditions. Four weanlings (Nos 53—56) inoculated at the same time with weanlings Nos 31—43 were placed in an isolated milieu where the danger of superinfection with PRV was excluded. The animals were examined 36, 57 and 75 days p.i.; neutralizing antibody was found in only one weanling in a consistently low titre. Reinoculation with the same small dose of PRV caused in the latter animal a great increase in the antibody titre. In the other two animals antibody formation was not detected even after the second low dose of BUK virus.

Some biological properties of viruses recovered from sc infected weanlings

Viruses isolated from sc infected weanlings retained in all cases biological properties characteristic of the inoculated vaccine strain BUK:

a) The plaque size under agar in CEC after 4 days at 37° C was 4 mm with all isolated viruses in their 1st passage in CEC (6 isolates from the SI 1—6 days p.i., 6 isolates from the ILN 1—6 days p.i. and 5 isolates from the CNS 2—6 days p.i.).

b) All the viruses isolated exerted in tube CEC cultures also in limit dilutions a fast and complete CPE with total degeneration of cultures 2—3 days p.i. After 1—2 passages in CEC, these viruses reached high titres ($\geq 10^7$ PFU/ml).

c) Rabbits inoculated with the isolates sc into the radix auriculae succumbed without any signs of pruritus (5 isolates from the CNS in the 1st passage in CEC, 3 isolates from the ILN and 2 isolates from the SI).

Discussion

The problem of multiplication or presence of vaccine PRV in the organism of vaccinated animals has been dealt with so far only by Rumanian authors (Bran *et al.*, 1963), although a number of live vaccines against Aujeszky's disease have been used for a long time in countries of central and south-east Europe. The authors mentioned found the vaccine virus only at the SI and in the corresponding LN 6—7 days p.i.; they did not examine the CNS or other organs. Since they expressed the results of the isolation experiments only as positive or negative, it cannot be concluded whether they had demonstrated residual virus or virus multiplication. Nevertheless, these results revealed that even in the case of their avianized virus which had undergone more than 200 passages in embryonated eggs there was no virus dissemination into the parenchymatous organs of vaccinated animals.

In this respect these results were fully confirmed in our experiments with the vaccine strain BUK (which, similarly to the commercial vaccine virus TK 200 of Dr. Žuffa, originates from the same virus, but after the first 100 passages in embryonated eggs had undergone a high number of passages

in CEC). However, using low vaccination doses we confirmed a significant, even though relatively poor, virus multiplication at the SI and in the corresponding LN. In the case of these doses, infective virus could not be detected 1 hour p.i. A similar phase of virus infection was found in the blood of guinea pigs after intravenous inoculation with vesicular stomatitis virus (Kosunen and Kääriäinen, 1966) and in mice inoculated sc with tick-borne encephalitis virus (Málková, 1968). The latter author called this phenomenon "the negative phase": it was followed by the detection of infective virus.

Simultaneously with the detection of PRV at the SI, its presence was established in the corresponding ILN. Since the infective titre of PRV in the ILN always was lower than that at the SI, it would seem that active virus multiplication did not proceed in this organ and that the virus was just passively transported there from the site of its multiplication. However, experiments with virulent PRV (Sabó *et al.*, 1968*a*, *b*; Rajčáni *et al.*, 1969) confirmed the multiplication of PRV also in the LN.

With both low and high infecting doses, we could detect the virus at the SI, in the ILN and CNS 1—8 days p.i., but not later.

The presence of PRV in the CNS after peripheral inoculation was observed with both variants of vaccine virus studied. The detection of PRV in the CNS was obtained in 18% of the animals, namely in 6 of 32 animals in which a positive result could be expected (excluding animals examined within the 1st day p.i. and later than 10 days p.i.). This proportion could be considered high from the point of view of the evaluation of vaccine innocuity; the significance of this finding needs detailed elucidation. In practice, when thousands of vaccinations were performed in field conditions by the standardized technique, involvement of the CNS which would be connected with striking clinical symptoms did not occur (Škoda *et al.*, 1966).

The present results offer evidence about the extent of differences between the attenuated vaccine strains and virulent viruses. The latter multiply in cases of natural or experimental infections to high titres in various organs of pigs, the mode of infection, the age of animal and the particular virus strain used probably playing an important role (Shope, 1935; Csontos *et al.*, 1962; Kojnok, 1962; Škoda and Žuffa, 1962; Albrecht *et al.*, 1963; Akkermans, 1963; McFerran and Dow, 1964*a*, *b*, 1965; Corner, 1965; Olander *et al.*, 1966).

The finding of the commercial vaccine virus TK 200 in the suprarenal gland and the dissemination of this virus by contact from vaccinated to non-vaccinated animals (Žuffa, 1963*b*) indicate that this variant possesses a higher virulence than the variant BUK.

The fact that pathological alterations were found only at the SI corresponds to the attenuation of the BUK virus and to the age of the infected animals. After intramuscular inoculation of a virulent virus Corner (1960) observed hyperaemia and necrotic foci at the SI. He also reported hypertrophy and necrotic foci in the suprarenal gland, which changes we could find only in one sucking pig infected with the TK 200 variant of vaccine virus. McFerran and Dow (1965) drew particular attention to a rather frequent virus detection in the suprarenal gland. With regard to sporadic

viraemia and a regular presence of virus in the lumbal spinal cord these authors concluded that PRV reaches the suprarenal gland via the neural routes from the spinal cord. Our finding supports this assumption.

PRV has been isolated from the blood in natural and experimental infections only rarely and exceptionally. McFerran and Dow (1965) reported only 2 positive isolations from more than 500 blood samples examined. McKercher *et al.* (1963) explained the similarly sporadic character of viraemia in the case of infectious bovine rhinotracheitis (this virus also belongs to the herpesviruses) by a passive transport of virus from the site of its primary multiplication to other organs by leukocytes. Hurst (1933) postulated that viraemia plays a role in the dissemination of PRV in the organism only in the case of the European (Hungarian) but not the American (Iowa) strain. Since the BUK strain was not found after sc inoculation in any parenchymatous organ of the pig, we interprete the unique finding of virus in the blood as a cellular viraemia, even though it lacks the experimental confirmation (Mims, 1964).

The blood route presumably plays no role even in the transfer of PRV to the CNS, where we could find the virus 2—6, most frequently 4—6, days p.i. PRV migrates from the SI to the CNS via the Schwann cells of nerve fibers innervating the area of primary virus multiplication or virus inoculation, respectively (Sabó *et al.*, 1968*a*; Rajčáni *et al.*, 1969).

The fundamental biological properties of the BUK virus did not change after passage through the organism of pigs.

Based on the present results is it possible to answer the question as to what is the mechanism of the immune response to the BUK strain vaccine? The dose of live virus particles itself, inoculated into the organism, may represent for the immunologically competent cells a sufficient stimulus for the establishment of immunity and concommitant induction of the antibody response or, in isolated cases (especially with low vaccination doses), at least for immunological modification of the organism without antibody response (Žuffa, 1963*a*). The fact that lower vaccination doses (below 10^4 PFU) induced a weaker immunological response leads us to assume that the BUK vaccine affects some animals as a specific antigenic stimulus (like the stimulus of an inactivated antigen of some microorganisms) and that virus multiplication at the SI does not substantially enhance the immunological reaction.

It should be emphasized that chemically inactivated PRV could not behave in the above-mentioned manner, because the antigenicity of PRV is so labile that it is destroyed by a minimal chemical attack, e.g. by treshold doses of formalin (Manninger, 1959; Žuffa, 1963*a*).

The development of new vaccines which started in the veterinary medicine mainly since 1945, has been steadily increasing. Statistically significant data on the innocuity and efficiency of new vaccines became a matter-of-course; similarly, detailed data on the fate of the vaccine virus in the organism of vaccinees should also be required for each vaccine. Such data appear essential for the evaluation and comparison of different vaccines.

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