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PERSISTENCE OF PERORALLY ADMINISTERED VIRULENT PSEUDORABIES VIRUS IN THE ORGANISM OF NON-IMMUNE AND IMMUNIZED PIGS

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Summary. — After peroral infection with 2×10^3—2×10^5 plaque forming units (PFU) of the virulent strain of pseudorabies virus, 6 weeks old non-immune weanlings showed no disease, although the virus was present in their tonsilar and oropharyngeal mucosa for 11—18 days post infection (p.i.) depending on the dose of virus inoculated. The appearance of specific neutralizing antibodies (NA) in serum of the animals also was directly proportional to the dose of infecting virus and their titres varied during the 10th—18th day p.i. After 18 days p.i., no virus could be demonstrated in the weanlings, either following intravenous injection of hydrocortisone (125 mg per animal), or by cultivation for 10—12 days of surviving tissue explants from tonsils. Nordid we succeed in provoking the infection either by overheating the organism, or by cold and diet stresses. The results suggested a direct correlation between the appearance of NA in the serum and the disappearance of virus from the pig organism. But such a relationship was not generally applicable. Animals first immunized by subcutaneous (sc) vaccination with the attenuated strain BUK and then challenged peroraly with 10^6 PFU of virulent virus maintained the virulent virus for 6—20 days p.i. On the day of challenge, the titre of NA in serum of the pigs varied from 1 : 8 to 1 : 16. Thus sc vaccinated pigs can be reinfected, maintain the virulent virus in tonsilar mucosa and release it therefrom.

Introduction

After an evaluation of the course of acute infection of sucking pigs with pseudorabies virus (Sabó *et al.*, 1968; Rajčáni *et al.*, 1969; Jamrichová and Škoda, 1969), we attempted to establish the infection in 6 weeks old pigs by the peroral route and to follow the persistence of pseudorabies virus in these animals. We employed older animals which usually survive the infection also in natural conditions.

We investigated first the duration of persistence of the virus in tonsilar mucosa and other of pigs experimentally infected by a natural route in relation to the formation of specific antibodies. Since we could assume the establishment of a latent infection, we tried to change it into a manifest form and to detect the virus after applying stress conditions. It was also of interest

to know whether the pseudorabies virus, applied by peroral route, is able to infect and multiply in the organism of pigs previously immunized sc by live attenuated vaccine BUK (Škoda *et al.*, 1964) and showing NA in serum.

Materials and Methods

Viruses. The virulent strain ČVOŠ was isolated from an ill pig by Škoda (1958, unpublished) and underwent 12 passages in chick embryo cell (CEC) cultures reaching a titre of 1.5×10^6 PFU in ml. The attenuated vaccine strain BUK was obtained by Škoda *et al.* (1964).

Experimental animals. We employed 6 weeks old pigs of the Slovak white breed weighing 8—10 kg. NA in animals before and during the experiment was assayed in the neutralization test in CEC as described previously (Sabó *et al.*, 1968).

Infection of animals was carried out by feeding them with 0.5 l of consume milk heated to 37° C and containing the respective virus dose (see Tables 1 and 2).

CEC cultures were grown in medium 199 supplemented with 10% of heated calf serum, penicillin (200 units/ml) and streptomycin (200 μg/ml).

Smears for virological examination were taken every 3 days p.i. from the throats (tonsils and oropharyngeal area) of the animals by means of sterile swabs which were then eluted each in 3 ml of medium 199 for 3 hours at 4° C. The eluate was centrifuged at 3000 rev/min for 10 minutes and 0.3 ml portions of the supernatant were inoculated into 4 parallel CEC monolayer tube cultures.

Evaluation of virus isolation experiments. The inoculated CEC cultures were incubated at 37° C for 7 days and examined for the cytopathic effect (CPE) daily. If the result of the virus isolation was negative (no CPE) or ambiguous, the medium from the culture was used for a second passage in CEC and in parallel inoculated sc to a rabbit. In the positive case, the rabbit showed strong pruritus and succumbed within 24—48 hours p.i. The virus could be isolated from its brain. Virus isolated either directly from swabs in CEC cultures of only after passage in the rabbit was identified in the neutralization test with a known hyperimmune serum against pseudorabies virus, or more rapidly by the immunofluorescence method.

Identification of pseudorabies virus by immunofluorescence (IF). CEC monolayer cultures grown on coverslips were infected with 0.2 ml volumes of media from cultures with positive CPE. Infected duplicate coverslip cultures were stained at 24 and 48 hours p.i. with specific conjugate against pseudorabies virus; two uninfected cultures served as controls, so that 6 coverslip cultures were needed for one complete test. If virus was present, there appeared a specific fluorescence of the antigen first in nuclei and, in later stages of infection, also in the cytoplasm. The fluorescent cells occurred as foci in the monolayer. The number of such cells increased in the course of infection so that finally the fluorescence was spread throughout the entire culture. The preparation of the conjugate and the method of staining of coverslip cultures were those described (Sabó *et al.*, 1968).

The dissected organs were examined for virus as described previously (Sabó *et al.*, 1968).

Explants from tonsils were prepared by the method of Israel (1962). The surviving tissue was maintained at 37° C in medium 199 containing 10% of heated non-immune pig serum, penicillin (200 units/ml), streptomycin (200 μg/ml) and mycostatin (200 μg/ml). The culture medium was changed daily and tested for virus presence by inoculation into CEC cultures.

Results

The persistence of pseudorabies virus in tonsils of non-immune pigs after peroral infection with the virulent strain ČVOŠ

The peroral infection of 15 weanlings with various doses of virus was carried out in three groups: 2×10^2 PFU (Nos 1—5), 2×10^3 PFU (Nos 6—10) and 2×10^5 PFU (Nos 11—15). The results of virus isolation experiments from the throat swabs and the dynamics of antibody formation are illustrated in Table 1.

Table 1. The persistence of the virulent ČVOŠ strain of pseudorabies virus in oropharyngeal mucosa of perorally infected pigs

Days p.i.		Virus dose									
		2×10^3 PFU					2×10^5 PFU				
		6	7	8	9	10	11	12	13	14	15
3	V	+	+	+	+	+	+	+	+	+	+
	NA	—	—	—	—	—	—	—	—	—	—
6	V	+	+	+	+	+	+	+	+	+	+
	NA	—	—	—	—	—	—	—	—	—	—
10	V	+	+	+	+	+	+	+	+	+	+
	NA	—	—	—	—	—	4	—	4	—	—
14	V	+	+	+	+	+	—	+	—	+	+
	NA	2	2	2	2	—	16	2	16	2	—
18	V	+	—	—	+	+	—	+	—	+	+
	NA	4	4	4	4	4	16	4	16	4	4
21	V	—	—	—	—	—	—	—	—	—	—
	NA	8	4	4	8	8	32D	8	16D	8	8
31	V	—	—	—	—	—		—C		—C	—C
	NA	64	16	16	16	16		16		16	16
42	V	—	—	—	—	—		—		—	—
	NA	128	16	32	32	32		32		16	16
57	V	—	—	—	—	—		—		—	—
	NA	64	16	32	16	16		16		8	8
67	V	—	—	—	—	—		—		—	—
	NA	64D	16D	32D	16D	16D		16D		8D	8D

6—15: Animal number.
V = Virus presence (+) or absence (—).
NA = Titre of NA in serum against 100 $TCID_{50}$ of virus; — no NA detected.
C = Treatment with cortisone.
D = Dissection.

The virus could not be isolated from any of the piglets Nos 1—5 and the animals did not respond to the infection by NA formation (therefore this group was omitted from Table 1). This fact showed that, even if the infection had taken, the virus did not substantially multiply and thus was not eliminated from the organism. All the other weanlings (Nos 6—15) showed the virus in swabs up to the 10th day p.i. The isolation was negative on the 14th day in animals Nos 11 and 13, and on the 18th day in animals Nos 7 and 8. The remaining animals (Nos 6, 9, 10, 12, 14 and 15) were positive for virus in throat swabs even on the 18th day p.i. The attempt to isolate the virus on the 21st day p.i. was negative in all animals.

To confirm that the absence of virus in throat swabs 18 days p.i. was due to the disappearance of virus from tonsils or from the whole organism of the animals, piglets Nos 11 and 13 were dissected at the time when three successive throat swabs were negative for virus. Virus was not isolated from the following organs: tonsils; nasopharynx; nasal mucosa; cervical, mediastinal, mesenteric and inguinal lymph nodes; oesophagus; trachea; lungs; liver; spleen; suprarenal glands; trigeminal nerve; brain stem; cerebral cortex; and cervical, sacral and lumbal spinal cord.

Nor was virus isolated from organs of weanlings Nos 6—10 dissected on the 67th day p.i.

A part of tonsils from the dissected animals was used for preparation of surviving cultures which could be maintained almost for 12 days. Samples of media taken daily from the cultures and examined for virus presence in CEC cultures gave negative results.

Attempts at virus detection in animals in the course of eventual latent infection after various stress treatments

After the disappearance of virus from throat swabs, animals Nos 12, 14 and 15 were subjected to cortisone stress in an attempt to activate the infection. The animals were given slowly 125 mg of hydrocortisone Spofa into the vena cava cranialis on the 13th day after the last positive virus isolation from swab. The weanlings reacted with a shock (fell down and showed tremor) from which they slowly recovered. Throat swabs were taken from the cortisone-treated animals for 36 days. During this period the animals were subjected three times to cold stresses (they were sprinkled with 12° C water for 10 minutes) and at approximately weekly intervals they were twice fed with a changed ration (what is considered as a stressing factor in pigs; in the present experiment they showed diarrhoea).

Thirty days after cortisone-treatment we attempted to evoke in the animals a stage which usually appears during their trasportation and overheating. Weanlings first underwent a physical strain in a summer weather (24° C) and then were kept in a little space in a poorly ventilated room for 3 days. They were fed only once a day with a small ration of liquid food.

Even under these conditions it was impossible to confirm the reappearance of virus in any of the throat swabs. Thirty-six days after cortisone treatment (67 days p.i.) the weanlings were dissected and their organs in the above-described extent assayed for virus in CEC; the results were negative. Tonsils were used for the preparation of surviving cultures; the culture medium was supplemented with 0.5 mg/ml of hydrocortisone. The cultures survived for 10 days; no virus was demonstrated in them.

While being positive for virus, the animals did not show any clinical signs of illness, elevated temperature or loss of appetite, but they responded immunologically by production of specific NA. NA appeared first in serum of weanlings Nos 11 and 13 on the 10th day p.i. in a titre of 1 : 4 (against 100 $TCID_{50}$ of virus); in weanlings Nos 10 and 15 they appeared only on the 18th day p.i. in a titre of 1 : 2. The titre of NA in serum increased during subsequent days and culminated on the 42nd day p.i. (1 : 16—1 : 128).

It was remarkable to observe that, provided the level of NA in serum was low (1 : 2—1 : 4), virus could be detected in tonsils in some cases, whereas after NA titres had increased tq 1 : 8 or more, the presence of virus in the animals could no more be demonstrated.

The persistence of virulent pseudorabies virus after peroral infection in the organism of pigs sc immunized with the attenuated vaccine strain BUK

The results mentioned above led us to test whether (1) the humoral antibodies after sc inoculation of the attenuated vaccine strain BUK protect the

Table 2. The persistence of the virulent ČVOŠ strain of pseudorabies virus after peroral infection of pigs se immunized with the attenuated strain BUK

Days p.i.		Animal No.									
		51	52	53	54	55	56	57	58	59	60
8	NA	2	2	4	4	2	2	2	4	2	2
14	NA	8	8	8	8	8	2	2	8	4	8
30*	NA	16	8	16	16	8	8	8	16	8	16
33	V	+	+	−	+	+	−	−	+	+	+
	NA	16	8	16	16	8	8	8	16	8	16
36	V	+	+	−	+	+	−	−	+	+	+
	NA	16	16	16	32	16	8	8	16	16	16
39	V	+	+	−	+	+	−	−	+	−	+
	NA	32	16	16	64	32	16	8	32	16	32
43	V	−	−	−	+	+	−	−	+	−	+
	NA	64	32	16	128	64	8	8	64	16	32
46	V	−	−	−	−	+	−	−	+	−	+
	NA	128	64	16	512	128	8	8	64	16	64
50	V	−	−	−	−	+	−	−	−	−	−
	NA	128	32	16	512	128	8	8	128	16	128
57	NA	64	32	32	256	64	4	4	64	8	128
64	NA	64	32	16	128	64	4	4	64	8	64
85	NA	64	32	16	128	64	4	4	32	8	64
92	NA	32	16	16	128	64	4	2	32	8	32

* On day 30, the animals were given per os 10^6 PFU of the ČVOŠ strain.
For other explanations see Table 1.

tonsilar mucosa against peroral infection with virulent virus and (2) how long does the virulent virus persist in the organism of an immunized pig, provided that the infection does take.

Ten animals were immunized by two doses, each containing 10^8 PFU of the BUK strain. The second vaccination virus dose was given 10 days after the first one. The formation of NA after vaccination is illustrated in Table 2.

Thirty days after the onset of vaccination, the weanlings were given perorally 10^6 PFU of the virulent strain ČVOŠ. Titres of NA in sera from the animals at the time of peroral challenge varried in the range of 1 : 8—1 : 16 (against 100 $TCID_{50}$ of virus). The oropharyngeal swabs taken 3 days after challenge were positive for virus in 7 animals, whereas the virus isolation in the remaining 3 animals was negative even at subsequent days. Nine days after challenge, the virus disappeared in animal No. 59 and after 13 days it did so in animals Nos 51 and 52. After 16 days virus was recorvered from swabs still in 3 weanlings (Nos 55, 58 and 60) and weanling No. 55 was positive even after 20 days. After 62 days virus could not be detected in the weanlings, even by the isolation experiments from individual dissected organs (as described above).

Animals, which showed the presence of virus in the oropharyngeal swabs for a certain period after peroral challenge, responded to the infection by

a marked increase of NA in their sera (titres up to 1 : 64—1 : 512 against 100 $TCID_{50}$ of virus). On the other hand, there was no increase of serum NA in animals which did not show the presence of the challenging virus (Table 2).

It was remarkable that the virus persisted in the organism of sc immunized animals after peroral infection even at a relatively high level of NA in serum (1 : 64—1 : 128 against 100 $TCID_{50}$ of virus).

Discussion

In studies on the pathogenesis of pseudorabies virus one has to consider the question as to whether the organism of a pig, when first infected, does become a long-term carrier of the virus and thus a source of infection for susceptible animals living in the same piggery. This view is based on the following facts: 1) infection with pseudorabies virus occurs in pigs early in their life, i.e. mostly in sucking pigs and, moreover, 2) pseudorabies virus belongs to the herpesvirus group (Andrewes, 1962) in which the long-term latent virus carrier state and repeated exacerbation connected with virus excretion are generally accepted despite the fact that the site of existence of the virus between exacerbations could not be unequivocally established.

The possibility to isolate pseudorabies virus from the upper respiratory tract of pigs by the use of swabs was reported by Kojnok (1965). He isolated the virus from the nasopharynx of 3.4% of clinically healthy pigs and from immunized sows. Wilke and Dannenberg (1968) tested organs of 200 clinically healthy fattening pigs originating from two endemically infested piggeries and isolated pseudorabies virus from tonsils, lungs and muscle tissue in 4.5% of the animals, while NA in serum were positive in 86.5% of the whole population examined. An exceptional case was reported by Nikitin (1965) who isolated pseudorabies virus from the lungs and liver of a pig 186 days after the last clinically manifest case of Aujeszky's disease in the piggery. It is difficult to prove in field conditions, however, whether this was actually a long-term virus carrier state or a fresh infection with a new strain of virulent virus.

The results of our experiments on the peroral infection of 6 weeks old non-immunized weanlings with the given doses of virulent virus revealed that the latter actually persists in the pig organism and is released for a rather long time, i.e. in our case at most up to the 18th day p.i., in spite of the fact that NA appear in low titres (1 : 2—1 : 4) in the serum earlier. These results support the view that peroral infection of 6 weeks old weanlings with the virulent strain of pseudorabies virus leads to its multiplication in the oropharyngeal, in particular tonsilar, mucosa and in this way to its active survival in these cells (see the positive fluorescence in epithelial cells of tonsils — Sabó *et al.*, 1968). A mere passive survival of virus cannot be involved, because the virus would be inactivated to a considerable extent by thermic and perhaps also other mechanisms, then eliminated, and thus unable to induce the formation of NA. This conclusion is supported by the observation that the peroral infection with a low dose of the virulent virus led neither to its detectable multiplication nor to the production of NA. Finally, though all

these proofs of active virus multiplication are essentially of an indirect character, it is worthwile to mention that apparently only a negligible part of the applied virus dose adhered to the tonsilar mucosa, whereas its bulk was carried with the food to the lower part of the gastrointestinal tract of the animal.

The present results would seem to support the long accepted view that the appearance of humoral antibodies leads to the elimination of virus from the organism. Data of a similar nature were reported for pseudorabies and bovine rhinotracheitis viruses (see McFerran and Dow, 1965). Nevertheless, the correlation between the presence of NA in serum and the absence of pseudorabies virus in the organism cannot be considered as generally valid. Our arguments are based both on the known experience with herpes simplex infection of humans and, mainly, on our experiments which proved the persistence of virulent pseudorabies virus in the oropharyngeal mucosa after peroral infection of sc immunized pigs, even under the conditions of a rather high level of NA in serum (1 : 64—1 : 128 against 100 $TCID_{50}$ of virus).

The presence and release of the virulent virus after peroral (natural) infection for 18 days in non-immunized pigs and for 20 days in immunized pigs can be considered as long enough to enable the infection of a number of susceptible animals from this source and in this way to secure the circulation of the virulent virus in a breed for an unlimited period of time. Under these conditions the circulation of virus in the breed may proceed unnoticed, especially if humoral antibodies in serum serve as a criterion, but also in view of the fact that older pigs undergo an inapparent disease, the latter becoming clinically manifest only from time to time, especially if the virus attacks the most susceptible population — the sucking pigs.

It appears that, in some cases, NA are unable to prevent not only the circulation of the virulent virus in the population, but also its dissemination in the organism, namely its penetration into the target organ — the central nervous system. In the period 1964—1968 we observed several cases of clinically manifest Aujeszky's disease in sucking pigs, which were dissected in a moribund state. Pseudorabies virus was isolated in these cases from brains, tonsils, lungs and cervival lymph nodes; NA titres in the sera from these animals varied from 1 : 32 to 1 : 128 (against 100 $TCID_{50}$). It is not excluded, therefore, that, besides the specific NA, there are also other, so far unidentified, mechanisms which play a role in the process of defense of the pig organism against pseudorabies virus infection. The elucidation of the latter mechanisms would substantially contribute to the understanding of the pathogenesis not only of this disease, but apparently also of the whole group of herpetic infections.

The persistence of the virulent pseudorabies virus in the population of pigs may be explained in at least two ways: either by a successive transfer of infection from one animal to another, which seems the most likely from our experiments, or by admitting the possibility of establishement of a latent infection, which was not excluded by Akkermans (1963). Or, even a simultaneous function of both these mechanisms might be assumed. We tried to prove the existence of a latent infection in our experiments by attempting

to induce the recrudescence of the infection by cortisone treatment, which is known as converting organisms poorly susceptible or resistant (with respect to their age) to susceptible ones (as was demonstrated e.g. in the case of infection of adult mice with coxsackievirus by Kilbourne and Horsfall, 1951). The application of cortisone did not enable the multiplication and release of pseudorabies virus and thus the chance of its detection in animals in which the virus could no more be found after the precious infection. We also failed to provoke the infection by some other stress factors; the subsequent attempts to isolate the virus from tissue explants cultivated for 10—12 days were also negative. We take into account, however, that the period of cultivation of the explants was too short to allow an absolute judgement about the presence or absence of a latent virus in the tissue.

In spite of the results so far obtained we do not dare to answer unequivocally the question as to whether the virulent pseudorabies virus, after several days of clearly demomstrated persistence in the organism of weanlings, does continue in persisting in a form which we cannot disclose or which cannot be converted to a stage of production of fully infective virus. We assume that further attention should be paid to studies on tissues in which the virus primarily multiplies after natural infection and from which it penetrates multiplies after natural infection and from which it penetrates into further tissues and organs of sucking pigs. In weanlings, it is the oropharynx, which the virus persists for some time in and is also released from.

From the present results we can conclude that even sc immunization of pigs with live attenuated virus will probably be unable to stop completely the circulation of the virulent virus in an endemic breed and the less to fully eliminate it from the population of pigs as has been originally assumed.

In this study we did not consider intentionally the importance of rats *(Rattus norvegicus)* in the ecology of pseudorabies virus in pig breeds and their significance as reservoir animals (Shope, 1935).

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