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DEVELOPMENT OF INHALATION RABIES INFECTION IN SUCKLING GUINEA PIGS

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Summary. — Massive extraneural multiplication of rabies virus in cells of the nasal mucosa of suckling guinea pigs preceded the involvement of the central nervous system (CNS). The primary pathogenetic importance of the nasal route in the establishment and further development of inhalation rabies infection was thus demonstrated. With respect to the assumed take and multiplication of virus mainly in neurosensory cells of the nasal mucosa, the inhalation infection may be considered as a peculiar form of neural infection. Late, erratic and quantitatively low findings of rabies virus in the lungs suggest that lung tissues play no important role in the primary replication of virus and in its spread into the CNS. The relatively early and persistent occurrence of virus in the kidneys seems to reflect haematogenous infection of the kidneys by virus resorbed at the site of its primary multiplication.

Introduction

In our previous work (Hronovský and Benda, 1969) on street rabies virus strain R-205 which had been adapted to primary dog kidney cell cultures we demonstrated the possibility of inducing a reproducible inhalation infection in laboratory rodents. Suckling guinea pigs proved to be the most susceptible. We selected them, therefore, for investigations on the mechanism of development of air-borne rabies infection. The results of our study are reported below.

Materials and Methods

The virus strains and the techniques of the inhalation infection were those described previously (Hronovský and Benda, 1969).

Distribution of virus in the organs in the course of inhalation infection was studied in suckling guinea pigs. Two animals were killed daily and samples were taken from various parts of their brains, spinal cords, salivary glands, nasal mucosa, trachea, lungs, thoracic nodes, livers, spleen, adrenals and kidneys. Ten per cent suspensions were prepared from a part of the samples, centrifuged, and virus in the supernatant fluids was titrated intracerebrally (ic) in 10 g mice (4 mice for each dilution). Before killing, blood was taken from the animals to detect viraemia.

Immunofluorescence (IF). The direct technique was used to demonstrate viral antigen, mainly in impression smears and less frequently in cryostat sections of samples frozen in liquid propane-butane. Hamster or guinea pig hyperimmune sera were obtained by a modified immunization schedule according to Lennette *et al.* (1965), using 4—5 increasing doses of the homologous brain suspension of the live CVS strain after preimmunization with the HEP Flury avirulent egg strain. Conjugation of the globulin serum fractions with fluorescein isothiocyanate (2.5 mg

amorphous FITC per 100 mg 4% protein solution) was carried out in borate buffer pH 9.0 for 20 hours at 4° C. Unbound FITC was removed by filtration through a Sephadex G-25 column. The working dilutions of the conjugates (1:8—1:16) were always prepared in the supernatants from the respective 20% normal organ suspension. The preparations were fixed for 1 hour in chilled acetone, stained for 30 minutes at room temperature, washed 3 times for 10 minutes in phosphate buffered saline and mounted in Tris-glycerol-saline stabilizing medium (Mrenová and Albrecht, 1966). As contrast stain, rhodamine sulphofluoride conjugated with bovine albumin (Noskov *et al.*, 1965) was used. After titration it was mixed with an equal volume of anti-rabies conjugate so that the final concentrations of either ingredient corresponded to the optimal working dilution. The preparations were examined in an ML-2 microscope equipped with an HBO-200 mercury vapour lamp. Micrographs were taken on ORWO 27 DIN film with exposures of 1—3 minutes.

Results

The results of investigations on the course of inhalation infection and virus distribution in the organs from infected suckling guinea pigs are summarized in Table 1.

For the first 5 days after inhalation infection with the absolute inhalation lethal dose ($>10^4$ ic mouse LD_{50}) of the R-205 strain, no virus was demonstrated in any of the organs, either by IF or infectivity assays. First positive findings by either method were recorded as late as on day 6, i.e. at about half of the incubation period, namely in the nasal mucosa. A relatively high concentration of infectious virus and massive occurrence of specifically fluorescent antigen were consistently demonstrated here also at later intervals. Virus in the CNS was detected one day later than in the nose. Detailed investigations by IF on the distribution of virus in brain tissues (in experiments not included into Table 1) showed that, in the early phases of CNS involvement, the antigen was markedly concentrated in the olfactory region of the brain (olfactory bulbs, hippocampus), but dispersed foci of inclusions were present also in cells of other cortical and extracortical parts of the brain, in the brain stem, cerebellum and cranial segments of the spinal cord. Virus in the spinal cord appeared mostly in elongated foci and the dynamics of virus titres suggested a descending spread of the infection.

In addition to the nose, extraneural multiplication of virus could be regularly demonstrated in the kidneys simultaneously with the first findings of virus in the CNS. During incubation, even in sick animals, the virus levels in the kidneys did not substantially increase and fluorescent antigen occurred in the form of infrequent scattered inclusions. Viral antigen in the form of small foci was found in the lungs and occasionally in cells of the trachea, but only at the end of incubation and during the symptom period. The findings of virus in the lungs were erratic and its titres were relatively low. The examination of rich materials from a number of experiments resulted in virus isolation from the lungs in only 20% of the animals with high levels of virus in the nose and CNS. Similarly erratic was the demonstration, only by IF, of virus in the salivary glands. No virus was found in the other organs (livers, spleen, adrenals and thoracic nodes) and blood. Attempts at IF detection and virus isolation from macrophages obtained from lung washings from infected guinea pigs failed.

Examples of IF findings are presented in Figs 1—8.

Table 1. Findings of rabies virus in organs of suckling guinea pigs in the course of inhalation infection

Organ examined		Days after infection											
		5		6		7		8		9		10	13
		*1**	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*12*	*16*
Brain	I	–	–	–	–	2.7	3.5	4.5	4.7	4.0	3.3	5.5	5.0
	FA	–	–	–	–	++	++	+++	+++	++	++	+++	+++
Medulla oblongata	I	–	–	–	–	1.5	2.3	3.3	4.0	2.5	2.5	4.8	5.7
	FA	–	–	–	–	+	+	++	++	++	++	+++	+++
Cervical cord	I	–	–	–	–	–	1.0	1.5	2.5	2.0	1.0	1.7	3.7
	FA	–	–	–	–	+	+	+	+	+	+	+	++
Thoracic cord	I	–	–	–	–	–	1.0	1.0	1.0	1.7	–	2.5	3.7
	FA	–	–	–	–	< +	+	+	+	+	< +	+	++
Lumbal cord	I	–	–	–	–	–	–	–	–	0.7	–	–	1.8
	FA	–	–	–	–	–	–	+	–	+	–	+	+
Nasal mucosa	I	–	–	1.5	1.3	2.7	4.0	4.5	4.3	2.3	2.7	3.0	3.2
	FA	–	–	+	+	++	+++	+++	+++	++	++	+++	+++
Trachea	I	–	–	–	–	–	–	–	–	–	–	0.7	–
	FA	–	–	–	–	–	–	–	–	–	–	< +	–
Lungs	I	–	–	–	–	–	–	1.5	–	–	–	2.3	–
	FA	–	–	–	–	–	–	< +	–	< +	–	+	–
Salivary gland	I	–	–	–	–	–	–	–	–	–	–	–	–
	FA	–	–	–	–	–	–	< +	–	–	–	+	< +
Kidneys	I	–	–	–	–	1.3	1.3	2.3	1.5	1.0	1.5	1.0	0.7
	FA	–	–	–	–	+	+	++	+	+	+	+	< +

* *1*, *2*, etc. — guinea pig numbers; animals Nos *9*, *10*, *12* and *16* were killed when showing symptoms.

I — Virus titres in log ic mouse LD_{50}/0.03 ml values; — means no virus detected (< 0.7).
FA — Findings of inclusions showing specific fluorescence: — no inclusions; < + single inclusions; + rare inclusions; ++ frequent inclusions; +++ numerous inclusions.

Discussion

Recent investigations on the distribution of rabies virus in naturally and experimentally infected animals showed that, in addition to the CNS and salivary glands, the virus occurs depending on the mode of infection also in muscle tissue, brown fat, lungs, kidneys and certain glands with inner secretion (Sulkin *et al.*, 1959; Dean *et al.*, 1963; H. N. Johnson, 1964). Apart from muscle tissue the involvement of other extraneural organs is considered to be a secondary one following previous multiplication of virus in the CNS (R. T. Johnson, 1965; Schindler, 1966).

The present results concerning the course of inhalation rabies infection in suckling guinea pigs confirmed that take of virus and its multiplication in the nasal mucosa is of basic importance in the establishment of this type of infection. Since nasal mucosa in laboratory rodents represents a highly developed perceiving olfactory region, consisting in addition to epithelial cells of a dense superficial network of sensory cells of the olfactory neuroepithelium, the inhalation rabies may be in principle regarded as a peculiar form of neural rabies. From the point of view of present knowledge, the demonstration of early and massive virus multiplication in the nasal mucosa, evidently preceding its multiplication in the CNS, represents a novel pathogenetic aspect and simultaneously offers evidence of the possibility of virus excretion from the animals by upper respiratory tract excreta. It may be assumed that after the virus had multiplied in the nose, there follows a rapid invasion of virus via the fila olfactoria into the olfactory region of the brain and probably also via other neural pathways (n. trigeminus, n. facialis) into the regions of their nuclei at the bottom of the 4th brain ventricle and of the medulla oblongata.

The participation of lung tissue as the primary site of virus multiplication in inhalation rabies cannot be excluded a priori. But, in contrast to the expectations and to the conclusions of Atanasiu (1965), the late, erratic and quantitatively low findings of virus in the lower respiratory tract and lung tissue suggest that the pulmonary route of virus spread into the CNS plays no important role in the pathogenesis of rabies in experimental animals infected by inhalation.

The early and regular occurrence of virus in the kidneys is of interest. The early time and especially the character of IF findings — the presence of scattered, usually isolated antigen corpuscles — suggests the probability of a haematogenous infection of the kidneys by virus resorbed into the blood stream from the sites of its primary multiplication in the nasal region. Our observations would seem to offer indirect evidence of the existence of viraemia which, of course, would lay below the level of sensitivity of the detection methods used. They also support the findings by some authors (Krause, 1957; Becker, 1963; Dean *et al.*, 1963) who offered indirect evidence of haematogenous transport of virus into certain organs early after infection. The role of viraemia in the pathogenesis of rabies, especially in the penetration of virus into the CNS, still remains a matter of discussion.

The sporadic and late occurrence of virus, demonstrable only by IF, in

the salivary glands is not surprising. It indicates a lowered affinity of virus to the salivary glands which has often been reported for wild strains that had undergone even a low number of mouse passages.

The fact that in a number of cases IF gave positive results when infectivity assay was negative, confirmed the frequently reported experience concerning the higher sensitivity of the IF method as compared with isolation experiments in rabies.

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Explanation of Photomicrographs:

Rabies virus antigen showing specific fluorescence in impression smears from organs of suckling guinea pigs infected by inhalation.

Figs 1, 2, 3, 5 and 8: guinea pig No. 7, killed without symptoms 8 days after infection.

Figs 4, 6 and 7: guinea pig No. 12, killed with symptoms 10 days after infection.

Fig. 1. — Olfactory region of the brain with typical spherical inclusions of various size. ×485.

Fig. 2. — Focal aggregation of antigen in the neck spinal cord. ×485.

Fig. 3. — Widespread involvement of cells in an impression smear from the mucosa of the nasal concha. ×485.

Fig. 4. — Detail of nasal mucosa. Diffuse fluorescence of a cell with numerous fluorescent inclusions. ×1090.

Fig. 5. — Another example of antigen in cells of the nasal mucosa. ×485.

Fig. 6. — Specific inclusions in lung cells. ×485.

Fig. 7. — An example of a comparatively poor finding of inclusions in the salivary gland. ×485.

Fig. 8. — Specific inclusions in an impression smear from the kidneys. ×485.