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COMPARATIVE STUDIES ON THE PROPERTIES OF VARIOLA VIRUS STRAINS I. CHARACTERISTICS OF CHORIOALLANTOIC MEMBRANE LESIONS AND PATHOGENICITY FOR CHICK EMBRYOS AFTER DIFFERENT METHODS OF INOCULATION

S. S. MARENNIKOVA, R. A. SHAFIKOVA

Research Institute of Viral Preparations, Moscow, U.S.S.R.

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Summary. — The study of 15 strain of variola virus revealed intraspecies differences in the pathogenicity for chick embryos (CE), the size of pocks and the intensity of multiplication in the liver of CE. The virus strains isolated from patients suffering from haemorrhagic forms of smallpox were usually more pathogenic for CE than those isolated from other forms of the disease. It was proved that variola and alastrim viruses can be distinguished from one another by estimating their multiplication in the liver of CE. The properties of strains isolated from patients suffering from lavly haemorrhagic variola did not exceed the range of variations found for the whole virus group.

Introduction

There have been only few reports on the existence of certain differences in the properties of variola virus strains (Murti and Shrivastav, 1957; Bedson *et al.*, 1963; Sarkar and Mitra, 1967).

The aim of the present study was a comparison of the properties of 15 strains of variola virus isolated from specimens taken from patients suffering from smallpox of different severity in various geographical regions of the world. The present results concern the pathogenicity for CE after different methods of inoculation (the Helbert's test included), and the size and morphology of the pocks formed.

Materials and Methods

The data on the virus strains studied are summarized in Table 1. Seven out of the total of 15 strains were isolated in CE by us just before beginning of the present experiments and the others were isolated earlier in this laboratory (Marennikova *et al.*, 1963). Specimens for isolations originating from cases of the early haemorrhagic variola were kindly supplied by Dr. Nanavati; Dr. V. S. Vishniakov supplied materials from Nepal, Dr. I. D. Ladnyi those from Kenya, and Dr. J. Noble specimens for isolation of alastrim virus.

Most of the virus strains investigated underwent 2—4 passages in CE at the beginning of the comparative studies. Suspensions from chorioallantoic membranes (CAM) prepared by the routine method (Marennikova, 1961) and stored frozen at —20° C in ampoules were used for inoculation of CE. The inoculation onto the CAM was carried out by the method of Westwood

Table 1. Characteristics of the strains tested

Strain	Place and year of isolation	Form of disease	Virus isolation		No. of CE passages
			Material*	Method**	
Ken-2-67	Kenya, 1967	Ordinary discrete	Crusts	CE	3
Ind-F-64	India, 1964	Confluent	BF	CE	2
Ind-MA-64	India, 1964	Confluent	VF	CE	4
Ind-K-66	India, 1966	Late haemorrhagic	VF	CE	3
Ind-D-66	India, 1966	Late haemorrhagic	VF	CE	3
Ind-B-67	India, 1967	Confluent	VF	CE	3
Ind-3a-67	India, 1967	Early haemorrhagic	Blood	CE	3
Ind-4a-67	India, 1967	Early haemorrhagic	Blood	CE	3
Nep-2-65	Nepal, 1965	Late haemorrhagic	Crust	CE	3
Nep-3-65	Nepal, 1965	Late haemorrhagic	PF	CE	3
M-T-60	Moscow, 1960	Ordinary discrete	VF	TC	2
M-A-60	Moscow, 1960	Variolois with single elements	VF	TC	2
M-S-60	Moscow, 1960	Ditto	VF	TC	3
M-N-60	Moscow, 1960	Ditto	VF	TC	3
A-E-56	Afghanistan, 1956	Ditto	VF	CE	10

* BF = bullous fluid; VF = vesicular fluid; PF = pustular fluid.
** CE = chick embryos; TC = tissue culture.

et al. (1957), into the allantoic cavity (AC) by the standard technique, and intravenously (iv) by the method of Beveridge and Burnet (1946). For inoculation onto the CAM or AC we employed 12 days-old CE and for iv inoculation 10 days-old CE. At least 8—10 CE were used for each virus dose. The volume of the inoculum was 0.1 ml with all routes of infection. The inoculated CE were observed for 7 days. With regard to our preliminary observations and available reports (Eichhorn, 1940; Beveridge and Burnet, 1946), indicating that a suspension of normal CAM may cause the death of CE during the first hours after iv inoculation, we did not take into consideration those embryos which had died within 24 hours post infection (p.i.).

The concentration of virus in the liver was estimated by the method of Helbert (1957). The character and the size of pocks on CAM was determined after 72 hours of incubation at 35° C. In order to measure the diameter of pocks, membranes carrying 10—20 pocks were withdrawn. The diameter of at least 200 pocks was measured for each virus strain by means of the binocular magnifier MBI-1. In estimating the average size of pocks, the halo of the infiltration was not considered.

The results of the pathogenetic studies were statistically evaluated by the χ^2-method (Belenky, 1963) and the average diameter of pocks was calculated after Rokitsky (1961).

Results

Inoculation onto the CAM

After inoculation by this route, all 15 strains caused a generalized infection of CE: pocks appeared on the heart, spleen, diaphragm and glandular stomach: besides that, haemorrhages in the myocard and muscular stomach, the involvement of the liver and lungs, and a hypertrophy of the spleen were observed.

The inoculation of a standard dose — 10^3 pock forming units (PFU) per ml — demonstrated the different pathogenicity for CE of the individual

Table 2. Characteristics of variola virus strains according to pathogenicity for CE, Helbert's index and pock size

Strain	Death rate (%) of CE inoculated			Virus level in the liver (PFU/g)	Mean diameter of pocks (mm)
	onto CAM with 10^3 PFU/ml	iv with			
		$10^{4.6}$ PFU/ml	$10^{3.6}$ PFU/ml		
Ken-2-67	0	50	20	2.7×10^7	n. t.
Ind-F-64	25	100	100	1.2×10^6	0.43
Ind-MA-64	25	100	100	8.0×10^5	0.50
Ind-K-66	37.5	100	75	3.6×10^7	0.56
Ind-D-66	25	100	100	3.9×10^7	0.49
Ind-B-67	25	100	100	1.4×10^7	0.32
Ind-3a-67	62.5	100	83	2.8×10^7	0.52
Ind-4a-67	57	100	100	6.6×10^7	0.49
Nep-2-65	62.5	100	100	2.8×10^7	0.47
Nep-3-65	57	100	75	n. t.	0.49
M-T-60	62.5	100	100	3.8×10^7	0.51
M-A-60	37.5	100	100	1.2×10^7	0.49
M-C-60	0	66	0	5.0×10^7	0.49
M-H-60	0	60	43	1.1×10^8	0.62
A-E-56	50	71	0	2.5×10^6	0.52
Butler*	n. t.	n. t.	n. t.	3.6×10^3	n. t.
B12C19*	n. t.	n. t.	n. t.	2.0×10^4	n. t.

* Alastrim virus strains.
n. t. = not tested.

strains: the proportion of dead embryos varied from 0 to 62.5% (Table 2). These results showed that the highest pathogenicity was displayed by 4 out of 6 strains isolated from a haemorrhagic form of variola, and in one of the Moscow strains (M-T-60) isolated from a discrete form of variola. The strain Ken-2-67 (from a case of discrete variola) and two strains isolated from a varioloid (M-S-60, M-N-60) proved to be apathogenic. Two other strains isolated from variolois cases (M-A-60, A-E-56) caused the death in 37.5 and 50.0% of CE, respectively. The differences observed in the pathogenicity of the strains were statistically significant.

The evaluation of the strains by the Helbert's test (after inoculation of 10^6 PFU/ml onto CAM) showed that the virus concentration in the liver varied from 8×10^5 to 1.1×10^8 PFU/g. Out of 10 strains causing death in 0—50% of CE, 3 strains (Ind-F-64, Ind-MA-64 and A-E-56) displayed the least amount of virus in the liver, while the remaining 7 strains did not differ from each other in this respect (Table 2).

After *inoculation into the AC* the infection developed less intensively. The virus titres (log ID_{50}) varied from 3.0 to 4.6, while after the inoculation onto the CAM from 6.5 to 7.0. Neither a dose of 10^3 PFU/ml, nor 10^6 PFU/ml killed the infected CE. By inoculating 10^6 PFU/ml or more of the virus, pocks appeared on the inner organs, but the embryos did not die.

The *iv infection* was carried out with doses from $10^{3.6}$—$10^{5.6}$ PFU/ml.

We observed that iv infection, like inoculation onto the CAM, led to the development of pocks on the CAM and the death of embryos. The onset of death of embryos after iv infection was similar to that after inoculation onto the CAM. Nevertheless, the involvement of the embryos themselves was considerably less expressed using the iv route of inoculation. If applied in a dose of $10^{5.6}$ PFU/ml, the death rate of CE was 100% with all the strains tested.

The reduction of the dose inoculated to $10^{4.6}$—$10^{3.6}$ PFU/ml enabled to demonstrate certain differences among the strains (Table 2). Namely, using $10^{4.6}$ PFU/ml, a rather lower death rate was observed with 3 out of 4 strains isolated from a variolois (M-S-60, M-N-60 and A-E-56) and with the strain Ken-2-67. It should be mentioned that all these 4 strains belong to the group causing the death in 0—50% of CE after inoculation onto the CAM. When applied in a dose of $10^{3.6}$ PFU/ml, two of these four strains behaved as fully apathogenic. The other strains (strain M-A-60, isolated from a varioloid, also included) killed the infected embryos in 75—100% of cases.

The size and morphology of pocks

The measurement of the diameter of pocks appearing on the CAM revealed its marked variation (0.2—1.1 mm) within one and the same virus strain. The calculation of average diameters enabled to establish certain differences among the individual strains. Most virus strains formed pocks with a diameter of 0.47—0.52 mm, but two strains (Ind-K-66 and M-N-60) formed larger (0.56—0.61 mm) and two other strains (Ind-B-67 and Ind-F-64) formed smaller pocks. It is noteworthy that those two strains causing larger pocks as well as those two causing smaller pocks belonged to that group of strains which killed 0—50% of CE after inoculation onto the CAM (Table 2).

Concerning pock morphology, all the strains could be characterized by causing white round solid pocks clearly demarcated from the surrounding parts of the membrane. Besides that, there was a small proportion of less solid pocks (2.5—11.0%), especially with the strains Ind-K-66, Nep-3-65, M-A-60 and M-N-60. Furthermore, the pocks formed by all strains differed from each other by the presence or absence of an halo of infiltration. The majority of pocks showed the aureole. The attempts to isolate virus clones from pocks possessing a different morphology (less solid pocks, pocks lacking the halo, small and large pocks) did not succeed: the subsequent passages revealed the typical as well as the above-mentioned different pocks.

Discussion

The present study was performed on 15 strains of variola virus, isolated from cases of haemorrhagic smallpox (6 strains); a confluent or discrete form of smallpox (5 strains) and a variolois (4 strains). The study of this group of strains revealed certain differences in practically all test criteria. Thus, a considerable variation was observed in the pathogenicity of the strains for CE after inoculation onto the CAM: most strains isolated from cases of haemorrhagic smallpox were more pathogenic. But this relationship was

not an absolute rule: two strains from cases of haemorrhagic form of the disease displayed a moderate lethality for CE; on the other hand, an enhanced pathogenicity was found with two out of nine strains isolated from cases of non-haemorrhagic disease (discrete variola and variolois). Wth regard to the two latter strains, it should be added that the patient who suffered from the discrete variola and from whom one of these strains was isolated, had been vaccinated in the childhood. The strain from the case of a varioloid, as distinct from all other strains, had undergone 10 passages in CE before entering the experiments, what fact itself could have contributed to an increase of its pathogenicity for CE, as shown in our preceding study (Marennikova, 1962).

Similar results were obtained in experiments on iv inoculation of virus. It should be mentioned that the strain isolated from the case of discrete variola in Kenya possessed a pathogenicity for CE similar to that of strains isolated from a variolois.

These results indicate that there exists a qualitative heterogeneity among variola viruses on the one hand and a known relationship between the severity of the disease in man and the pathogenicity for CE for a particular virus strain on the other. These findings are in accordance with those of Sarkar and Mitra (1967). A correlation between the course of disease and the character of the involvement of the CAM (haemorrhages) and also the enhanced pathogenicity was reported by Murti and Shrivastav (1957) and Marennikova (1962).

The fact that none of the strains tested, even in large doses, killed CE after inoculation into the AC confirms a previously observed phenomenon of the reduced ability of variola virus to multiply after inoculation by this route (Marennikova, 1962).

The estimation of the virus in the liver of CE inoculated onto the CAM (Halbert's test) revealed a high level of variola virus multiplication and an insignificant variation among various strains in this respect. Similar experiments with alastrim virus revealed that this virus multiplies in the liver to a lower extent than variola virus. This demonstrates a possibility to employ Helbert's test for the differentiation of these related viruses. Our findings differ from those of Sarkar and Mitra (1967) who reported a dependence of the intensity of virus multiplication in the liver on the degree of the pathogenicity for CE. It is not possible at present to find a plausible explanation of these controversial results. We can only mention that our results on the multiplication of virus in the liver fit those of Helbert (1957), who was the first to describe this phenomenon.

Finally, the present results disclosed a variation in the size of pocks formed on the CAM of infected CE.

Summarizing the present data, it can be stated that variola virus, despite of a remarkable stability of its properties, possesses a certain intraspecies variability, like all other viruses.

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