

THE ROLE OF ALVEOLAR MACROPHAGES IN INHALATION INFECTION OF RABBITS WITH VACCINIA VIRUS.

II. EXPERIMENTS WITH MEDIUM AND SMALL DOSES OF VIRUS

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Summary. — After sublethal inhalation infection of rabbits with a neurotropic strain of vaccinia virus and in the early stages of infection elicited with a few lethal doses of the same strain, viral antigen was demonstrated by immunofluorescence in bronchial washings exclusively in macrophages. Only at the later intervals of lethal infection a few ciliated epithelium cells with specific fluorescence in their cytoplasm could be detected in the washings. These findings were compared with the results of fluorescent antibody (FA) tracing in the lungs of infected animals. The present results differed from those after inhalation of large doses of virus and might serve as a basis for further studies about the role of alveolar macrophages in poxvirus infections.

Introduction

In our preceding paper (Daneš *et al.*, 1969) we reported about the results of inhalation infection of rabbits after a large dose of two different, the neurotropic and dermatropic, strains of Poxvirus officinale. We concluded that the alveolar macrophages did not significantly participate in the development of the infection process early after virus inhalation. With the aim to obtain more exact information about this problem, small and medium doses of the neurotropic strain were used in the aerosols. The results reported below offered evidence of an active role of the so-called alveolar macrophages in the development of sublethal and subacute poxvirus inhalation infection.

Materials and Methods

The neurotropic strain of vaccinia virus was the same as used previously (Daneš *et al.*, 1969). It was passaged and titrated in rabbit kidney (RK) cells prepared according to Rappaport's (1956) technique. The results of virus titration are expressed in TCID₅₀/ml values.

The nebulization of virus into the flow chamber, the detection of aerosol and calculation of the virus dosis breathed in by the animals, the preparation of bronchial washings and cellular smears and the FA staining differed from the procedures described previously only in some details. Instead of pentobarbital narcosis, the rabbits were killed by air embolisation into the ear vein and instead of the use of rhodamine sulfofluoride-labelled albumin an Evans blue solution (1 : 50000) was used to counterstain the FA-stained preparations (Nichols and McComb, 1962). Some of the cryostat lung sections (10–20 μ thick) stained with FA were photographed and

then rinsed in saline, fixed in formol solution and restained with haematoxylin and eosin to identify the fluorescent structures by histological examination. Some specimens, in addition to those destined for immunofluorescence, were treated separately for histological examination.

Results

In four experiments groups of 10—15 rabbits were exposed to graded doses of the virus aerosol. At given intervals after infection (p.i.), only one

Table 1. Detection of vaccinia virus (neurotropic strain) in materials from rabbits infected by inhalation of various doses of virus

Exp. No.	Dose of virus inhaled (TCID ₅₀)	Average death time of rabbits (days)	Days p.i.	Levels of virus in			Immuno-fluorescence ¹⁾
				lung washings		Opposite lung (TCID ₅₀ /g)	
				supernatant (TCID ₅₀ /ml)	TCID ₅₀ /10 ⁶ cells		
1	6000	7.6	0 ²⁾	0	0	0	—
			2	60	120	5 × 10 ³	(+)
			4	1,430	3,500	5 × 10 ⁴	+
			5	450	500	15 × 10 ⁴	++
			7	1,500	5,500	1 × 10 ⁵	++++ (*)
2	5500	8	0	0	0	0	—
			2	Traces	30	15 × 10 ²	—
			4	150	1,500	5 × 10 ⁴	+
			5	150	1,800	16 × 10 ⁴	+
			6	400	15,000	16 × 10 ⁵	+++ (*)
			7	1,500	35,000	35 × 10 ⁵	++++ (*)
3	2000	9.5	0 2	0	0	0	—
			4	Traces	20	15 × 10 ²	—
			5	Traces	230	15 × 10 ³	(+)
			6	150	150	2 × 10 ⁴	+
			7	35	225	15 × 10 ⁴	++
			8	500	1,500	25 × 10 ⁴	+++ (*)
			9	750	2,250	Not done	+++ (*)
			4	1000	Animals survived	0 3	0
4	0	0	30			—	
5	Traces	30	500			(+)	
6	15	300	300			(+)	
10	Traces	50	500			(+)	

¹⁾ Findings in cells from lung washings. Each preparation contained the indicated numbers of specifically fluorescent cells per 10—20 thousand elements:

— No fluorescent cells

(+) Very few fluorescent macrophages

+ Up to 5 fluorescent macrophages

++ Up to 20 fluorescent macrophages

+++ Up to 100 fluorescent cells

++++ More than 100 fluorescent cells

(*) In these samples also single fluorescent ciliated epithelium cells were found in addition to frequent macrophages.

²⁾ 0 means 1 hour p.i.

animal was killed. The results, summarized in Table 1, showed that the average survival time of rabbits increased with decreasing virus doses in the aerosol; a dose of 1000 TCID₅₀ was no more lethal. The titration of virus in the specimens, despite of a considerable variation of the results, also pointed to a prolongation of the latent phase of the infection. In general agreement with these results were also the numerous positive FA findings in the cells of bronchial washings.

The results obtained by the examination of materials from the last experiment (sublethal doses) appear important. In animals killed 5, 6 and 10 days p.i., only small amounts of virus were found and the FA assay of the smears from bronchial washings revealed the fluorescence only in a few macrophages, usually containing cytoplasmic inclusion bodies. Similarly, in samples, taken at the early intervals in all the other experiments, the viral antigen was observed exclusively in macrophages.

Epithelial cells containing the viral antigen were seen only in smears, coming from the late stages of lethal infection. For example in experiment 2 (virus doses of about 5500 TCID₅₀) two ciliated epithelium cells were detected in the smear from the bronchial washing on the 6th day p.i. and a small group of epithelial cells on the 7th day revealed a granular fluorescence. Similarly, only a few fluorescing epithelial cells were found in the washings from rabbits at the terminal intervals in experiments 1 and 3.

The differences between the present and previous findings (Daneš *et al.*, 1969) concern the number and type of cells in the washings as revealed by Unna-Pappenheim staining. Only small amounts of epithelial cells ranging from a few cells in the initial phase of infection to 5—8% of the cells at the late phase, were counted in the majority of the washing samples. The occurrence of polynuclear leucocytes and lymphocytes was also low and irregular.

Examination of the lung cryostat sections contributed to a more exact explanation of these findings.

In rabbits killed 2, 4, 6 and 7 days p.i. (experiment 2), scattered larger foci of specific fluorescence were found to involve the medium sized and small bronchi, the adjacent arterioles, the peribronchial and periarteriolar alveoli and respiratory bronchioles. Around these larger foci smaller groups of fluorescent cells were seen in alveoli and bronchioles. The FA findings in transversely cut bronchioles indicated a segmentary involvement of the mucosa. The histological picture of the nodules did not differ from findings described by Lancaster *et al.* (1966). In general, the development of histological lesions was relatively slight as compared with the more conspicuous immunofluorescence, especially at the early stages of infection. The round-cell infiltrate of the nodules rarely showed positive fluorescence in this phase.

Important were the findings in the rabbits killed 6 and 10 days p.i. in experiment 4 (sublethal infection). In these animals small, rarely scattered foci of fluorescence were detected in the alveoli and respiratory bronchioles. Fluorescence in the bronchial respiratory epithelium could not be definitely demonstrated. After staining of the sections with haematoxylin and eosin,

round cell infiltration of the interalveolar septa and distinct necrosis of the bronchiolar lining were observed.

Examples of the findings in the smears from washings and in lung sections are illustrated in Figs 1—14.

Discussion

The retarded respiratory lethal neurovaccinial infection of rabbits differs from the infection after large virus doses in several points. Regarding the aim of our work, the most important difference is the prevalence in the bronchial washings of alveolar macrophages containing viral antigen. Only in the latest stages of infection a few ciliated epithelium cells were found to contain viral antigen, although its occurrence in the bronchial respiratory epithelium was unequivocally demonstrated by the FA technique in accordance with Lancaster *et al.* (1966). The differences became more striking in case of sublethal infection. In this, the viral antigen occurred exclusively in macrophages (although only in a few cells), while in the lung tissue the small foci of specific fluorescence involved the alveoli and respiratory alveoli. Bronchial epithelium was not a place of primary manifestation of sublethal neurovaccinial infection process in rabbits. The ciliated epithelium cells are probably able to eliminate the small amounts of impacted aerosol by mechanical way. This mechanism cannot act in the respiratory bronchioles and alveoli, where the penetrating viral particles come into direct contact with the cells of epithelial lining.

Considering the role of alveolar macrophages in the infection process (Mims, 1964) we would like to point out two important circumstances. The first is the origin of the macrophages in bronchial washings. These cells may be pneumocytes, sloughed off from the alveolar lining, endothelial cells of the capillaries, blood monocytes, histiocytes or other cells. Secondly, we do not yet exactly know, in which of the cells mentioned the virus does multiply and in which it does not, and if any of these cell possess an ability to destroy the virus, like what is known with bacteria. Nor *in vitro* experiments help us to clear this question (their results only confirmed the ability of the majority of macrophages to reproduce vaccinia virus antigen and also the identity of morphological FA findings on macrophages, obtained in both *in vitro* and *in vivo* experiments). All these facts should be taken into account in interpreting the conclusion that the alveolar macrophages participate in the development of inhalation poxvirus infection and that their contribution becomes more evident on infection with small or sublethal virus doses.

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Explanation of Photomicrographs

- Fig. 1.* Specific fluorescence of the cytoplasmic inclusion bodies in macrophages from bronchial washing, 6 days p.i. with a medium virus dose. Approx. $\times 1050$.
- Fig. 2.* As *Fig. 1*, but 7 days p.i. with a small lethal virus dose.
- Figs 3—8.* Lung sections stained by the FA method; lethal doses of virus.
- 3* — Fluorescent foci around arterioles near to a small artery. The arteriolar elastica show nonspecific fluorescence; 4th day p.i. Approx. $\times 200$.
- 4* — Fluorescence of interalveolar septa on the periphery of a nodule; 4th day p.i., approx. $\times 400$.
- 5* — Specific fluorescence around the wall of a larger artery; 6th day p.i., approx. $\times 400$.
- 6* — Scattered fluorescence in a bronchiole and its surrounding; 4th day p.i., approx. $\times 400$.
- 7 and 8* — High power view on the bronchial lumen with groups of fluorescent cells (*8*) and on the bronchial epithelial lining with segmentary involvement (*7*); 4th day p.i., approx. $\times 800$.
- Figs 9—12.* Lung sections stained by the FA method and restained with haematoxylin and eosin; 2nd day after inhalation of medium lethal doses of virus. Approx. $\times 600$.
- 9 and 10* — Fluorescence in the area of bronchiolar branching (the epithelium of one bronchiole is partially necrotic) and marginal necrosis of a developing nodule.
- 11 and 12* — Primary foci of fluorescence occurring in bronchioles and the developing histological changes.
- Fig. 13.* Fluorescent in individual cells of a smaller bronchus near to a well developed nodule; 6th day p.i. with medium lethal dose. Approx. $\times 400$.
- Fig. 14.* Three primary foci of specific fluorescence around a not identified lung structure; 6th day p.i. with a sublethal dose. Approx. $\times 600$.