Acta virol. 13 : 521—526, 1969

THE ROLE OF ALVEOLAR MACROPHAGES IN INHALATION INFECTION OF RABBITS WITH VACCINIA VIRUS. I. EXPERIMENTS WITH LARGE DOSES OF VIRUS

L. DANEŠ, R. BENDA, J. KRUML, A. JELÍNKOVÁ

Military Institute of Hygiene, Epidemiology and Microbiology, Prague; and Department of Immunology, Institute of Microbiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

Received December 27, 1968

Summary. — Rabbits were infected with large doses of aerosols of two Poxvirus officinale strains possessing different virulence. At intervals, cells and fluid of the bronchial washings and the lung tissues were tested for virus. Multiplication of the virus in the lung tissue, before its occurrence in the washings, was repeatedly proved. At the stage of developed disease, viral antigen was found in epithelial cells, which sloughed off into the washing. At later intervals after infection viral antigen was also visualized in a part of free alveolar cells, apparently macrophages. Histological and fluorescent antibody (FA) examinations revealed the production of peribronchial and periarterial lesions.

Introduction

We are not aware of any report about the role of alveolar macrophages in viral inhalation infection. It is not yet clear as to whether alveolar macrophages act in transport and replication of the inhaled virus in a way like that formulated by Mims (1964) for similar free cells, or whether they take part in defense mechanisms of tissue clearing like those known in bacterial aerosol infection.

With the aim to obtain information about these problems, we carried out experiments on inhalation infection of rabbits with vaccinia virus.

Materials and Methods

Poxvirus officinale, strain "dermolapina", continuously passed in the rabbit skin, was obtained in 1962 from the Institute of Viral Preparations, Moscow. The dermal scrapings, harvested on the 4th day after skin scarification, were used in the form of a 10% suspension. The strain did not cause death of rabbits after intradermal injection and only irregularly penetrated into the blood, regional lymph nodes, lungs and liver. The neurotropic "neurolapina" strain was obtained from the same Institute. It has been maintained by intracerebral passages in rabbits and was used in the form of brain suspension. It killed rabbits after intracerebral and intradermal inoculation within 4—10 days depending on the dose used. It regularly penetrated into the blood and multiplied in numerous visceral organs producting necrotic lesions.

Chinchilla rabbits from the farm Mezno, weighing 2500—3000 g, were used.

Virus titrations were performed in HeLa cells. Tube cultures were infected 24 hours after seeding. After incubation for 48 hours at 37° C chick erythrocytes were added and the number

of foci of haemadsorption was counted. The results were expressed in plaque forming units (PFU) per 1 g of organ weight or for 1 ml of fluid volume.

Inhalation infection. The animals infected in a steel continuous flow chamber of 600-l volume. The aerosol was developed by an atomizer to achieve droplets of about 1 μ diameter. The air flow was 100 l per minute, the temperature 20—21° C and the relative humidity 74—78%. The calculation of the virus dosis was described by Daneš *et al.* (1962).

Autopsy of the animals was carried out after exsanguination in deep pentobarbital anaesthesia. The main left bronchus was clamped and the upper part of the trachea was also compressed. After introducing a syringe into the trachea, the right lung was washed out with 20 ml of phosphate buffered saline at pH 7.2. The solution was allowed to remain in the long for a few seconds, only to rinse away the sloughed cells, but not to damage the rest of respiratory epithelium.

Part of the left lung was fixed in 10% formalin for histological examination, part of both the left and right lung was kept on dry ice for virus titration and part of the left lung was evacuated and frozen for FA examination. In the washings, we determined the cell counts per ml and after centrifugation at 2000 rev/min for 10 minutes, the supernatants and the pellets with known amounts of cells were used for virus titration.

Smears were also prepared from the pellets for differentiation of the cells (May-Grünwald-Giemsa) and for FA assay.

FA technique. The direct method was applied to acetone-fixed cryostat sections or smears. The conjugate of specific rabbit immuno-globulin with fluorescein isothiocyanate (FITC) (Noyes and Watson, 1955) had a FITC : protein ratio of 8×10^{-3} and was used in a working dilution of 1 : 16. A 1 : 8 dilution of the conjugate was mixed with an equal volume of a twice concentrated working dose of rhodamine sulphofluoride-labelled albumin (Noskov *et al.*, 1965). The procedures used in staining and mounting of the preparations, fluorescence microscopy and photomicrography were those described by Benda *et al.* (1968).

The histological preparations were stained with haematoxylin and eosin and by the van Gieson method.

Results

Two groups of 16 rabbits each were infected by inhalation with either the neurotropic or the dermotropic strain of vaccinia virus. According to the calculations, the animals inhaled from 40600 to 48700 PFU of virus. Two rabbits of either group died 6 days after infection (p.i.). The materials from these dead animals was not examined.

The amounts of infective virus were determined in both the left and right lungs, and in the supernatant and cellular pellet of the bronchial washings at 2, 24, 72 and 144 hours p.i. The results are summarized in Table 1. Only traces of virus were detectable in some samples taken 2 hours p.i. in either group. At 24 hours, the virus multiplied well in the lung tissue, but only traces were found in one of the washings. Marked differences occurred between the two strains at 72 hours p.i. The neurotropic strain was isolated from all specimens examined. The dermotropic strain was found only in some of the washings and in the lung tissue it did not reach levels as high as the neurotropic strain. At the last interval (144 hours) the virus was isolated from all samples. Especially the neurotropic strain multiplied to high levels in the lung tissue. In all virus-positive washings, the virus content in the cellular fraction was higher than in the supernatant. In rabbits Nos 7 and 9 the virus was found only in the pelleted cells, but not in the supernatants of the washings. In 11 out of the 28 animals examined, the virus was detected only in lung tissues, the washings being negative. The levels of virus found in the left and right (washed) lung varied somewhat, but they were not significantly influenced by the washing procedure.

Table 1. Presence of the neurotropic and dermotropic strain of Poxvirus officinale in the pelleted cells and supernatant fluid from bronchial washings and in the lungs at different intervals p.i.

Virus strain	Hours p. i.	Rabbit No.	Virus titre (PFU)* in			
			bronchial washing		lung	
			cells	supernatant	left	right
Dermotropic	2	1	neg.	neg.	Traces	neg.
		2	neg.	neg.	56	Traces
		3	neg.	neg.	neg.	neg.
	24	4	neg.	neg.	3970	5100
		5	neg.	neg.	9470	9620
		6	neg.	neg.	730	530
		7	Traces	neg.	100	560
	72	8	neg.	neg.	1100	1200
		9	1000	neg.	10000	7000
		10	500	360	1650	1050
	144	11	380000	30500	25000	35000
		12	55000	35000	27200	20000
		13	16000	5040	500000	450000
		14	18000	5050	47800	17500
Neurotropic	2	15	neg.	neg.	neg.	neg.
		16	neg.	neg.	neg.	neg.
		17	neg.	neg.	neg.	neg.
	24	18	neg.	neg.	Traces	neg.
		19	neg.	neg.	6100	5130
		20	neg.	neg.	116	103
		21	neg.	neg.	105	465
	72	22	340	180	16000	5500
		23	29000	6000	11000	12000
		24	2850	2250	21000	21300
		25	1250	345	64000	73000
	144	26	390000	65300	950000	970000
		27	158000	59000	3200000	2050000
		28	352000	36500	2120000	2050000

* The titres are expressed per 10^6 cells, 1 ml supernatant fluid or 1 g lung tissue, respectively.

The cell counts in washings from different animals varied widely. The highest concentration of cells was present in the washings obtained after 144 hours.

The cell population found in the washings showed a gradual decrease in the numbers of lymphocytes, monocytes and macrophages. After infection with neurotropic strain, the quantity of epithelial cells markedly increased, while polynuclear granulocytes prevailed in case of the dermotropic strain. The types of cell found in the washings are demonstrated in Figs 1—7.

The smears and frozen lung tissue sections were examined by the FA technique. No specific fluorescence was found in the smears or lung tissue either at 2 or at 24 hours p.i. A few macrophages showed positive fluorescence in the smears after 76 hours. The ratio of positive cells to those containing

no viral antigen was about 1 : 300. The cytoplasm of some ciliated epithelial cells also displayed granular or homogeneous fluorescence.

At 144 hours after application of the dermotropic strain, a few macrophages were found to harbour viral antigen at the margin of their cytoplasm. At the same interval after inhalation of the neurotropic strain, fluorescence in the cytoplasm of macrophages was more abundant. The whole cytoplasm of the ciliated epithelial cells showed a specific fluorescence, but the number of positive cells was greater after inhalation of the neurotropic (60.4%) than the dermotropic (0.8%) strain. The fluorescence of viral antigen in the cytoplasm of alveolar macrophages and respiratory epithelium cells is demonstrated in Figs 8—16.

No specific fluorescence was found in lung sections 2 and 24 hours p.i. At 72 hours after the inhalation of the dermotropic strain the results were still negative. In animals infected with the neurotropic strain, several relatively large foci of specific fluorescence were found around bronchioles and arteries. At 144 hours a similar picture was found in animals infected with the dermotropic strain. At the same interval after infection with the neurotropic strain, a widespread specific fluorescence was found in cells of the alveolar walls and in free intraalveolar cells.

Only slight histological changes were seen at 2 hours p.i. with both virus strains. The interalveolar septa were occasionally widened and slightly infiltrated with mononuclears. Here and there lymphocytes penetrated through the basement membrane into the intercellular spaces of the bronchial epithelial lining.

At 24 hours p.i. the number of mononuclears in the interalveolar septa increased. Especially after inhalation of the neurotropic strain the picture became more varied. Some alveoli were intact, others showed widening of the septa or were filled with pale badly stainable fluid and fibrin network. The respiratory epithelium of the bronchioles showed necrosis of different extent. In the parts of the epithelium, which did not undergo necrosis, signs of cellular proliferation occurred. The walls of bronchial artery branches, near to the bronchioles changed by inflammation, also showed partial necrosis.

At 72 hours after the administration of the dermotropic strain, changes similar to those described above developed. In rabbits, infected with the neurotropic strain the inflammatory changes revealed progression in bronchioles, small bronchi as well as arterial walls. The most developed histological changes in the bronchioles occurred on the 6th day p.i. (Figs 17 and 18). The alveolar septa, however, were not involved to a larger extent and the focal distribution of the interalveolar infiltration was similar to that at the earlier stages of the infection. The necrotic changes of the arterial walls were more widespread and the adventia of the involved vessels was infiltrated with lymphocytes (Figs 19 and 20).

The lymph nodes in the pulmonary hilus showed maximal proliferation of germinal centers and numerous tingible bodies at 72 hours after inhalation of the neurotropic strain. The number of cells in marginal sinuses of nodes increased with progressing infection. Among cells in the sinuses, lymphocytes

prevailed, but also macrophages, plasmatic cells and polynuclear granulocytes could be distinguished.

The mucous membranes of larger bronchi and of the trachea revealed no pathological changes as compared to healthy animals. Only in the stroma of the tracheal mucous membrane a few lymphocytes, plasmocytes and mononuclears were detected 6 days p.i. with the neurotropic strain.

Discussion

The results of our present experiments, in which great amounts of Poxvirus officinale were used in the aerosol, did not sufficiently elucidate the role of the alveolar macrophages in the early stages of vaccinia infection of the highly susceptible rabbits. The virus was shown to multiply in lung tissue before it was demonstrated in the cells of bronchial washings. Despite of the quantitative differences in the number of cells in the specimens (1 g of tissue and 10^6 cells in the pellet), the absence of virus in cells of the washings should be regarded as important. The increase in the virus level in the washings at later intervals p.i. was connected with the occurrence of epithelial cells (some of which, in particular after administration of the neurotropic strain, were identified as typical ciliated cylindrical cells), and the viral antigen was revealed by immunofluorescence in these cells. The number of polynuclears in the smears was also high. This was in agreement with the histological findings showing at the development of inflammatory lesions in bronchioles.

The bright specific fluorescence in the macrophages at the later stages of infection might be explained by replication of the virus in these cells. It cannot be excluded, however, that these cells were pneumocytes, which sloughed off from the alveolar walls into the washing fluid.

Important are the results of a control experiment (unpublished), in which rabbits were infected with a lower dosis of the neurotropic strain of vaccinia virus (30000 PFU). These animals also succumbed on the 6th day, but in the smears from their bronchial washings macrophages prevailed also at the later stages of infection (approximately 90% of cells in the pellet). This finding led us to study the infection process after lower, gradated doses of virus. These experiments will be described in a subsequent communication.

The results of virological, immunofluorescent and histological examinations suggest that, during the first 24 hours, the macrophages did not substantially participate in the infectious process following inhalation of large amounts of two strain of Poxvirus officinale possesing different virulence. In agreement with the conclusions of Westwood *et al.* (1966) and Lancaster *et al.* (1966), our results confirmed the involvement of the vessels.

References

Benda, R., Noskov, F. S., Daneš, L., Serbezov, V. S., and Goldin, R. B. (1968): Visualization by immunofluorescence of elementary bodies and their aggregates in materials from skin lesions in poxvirus infections. *Acta virol.* **12**, 229–232.

Daneš, L., Libich, J., and Benda, R. (1962): Experimental air-borne infection of mice with tick-borne encephalitis virus. *Acta virol.* **6**, 37—45.

Lancaster, M. C., Boulter, E. A., Westwood, J. C. N., and Randles, J. (1966): Experimental respiratory infection with poxviruses. II. Pathological studies. *Brit. J. exp. Path.* **47**, 466—471.

Mims, C. A. (1964): Aspects of the pathogenesis of virus diseases. *Bact. Rev.* **28**, 30—71.

Noskov, F. S., Boldasov, V. K., Goldin, R. B., Yermakov, N. V., and Volkova, L. A. (1965): Contrast method of immunofluorescent demonstration of adenoviruses in cultures of guinea pig kidney cells (in Russian). *Vop. Virus.* **10**, 613.

Noyes, W. F., and Watson, B. K. (1955): Studies of the increase of vaccine virus in cultured human cells by means of the fluorescent antibody technique. *J. exp. Med.* **102**, 237.

Westwood, J. C. N., Boulter, E. A., Bowen, E. T. W., and Maber, H. B. (1966): Experimental respiratory infection with poxviruses. I. Clinical, virological and epidemiological studies. *Brit. J. exp. Path.* **47**, 453—465.

Explanation of Photomicrographs:

Figs 1—7. Differentiation of cells in smears from bronchial washings. Neurotropic strain of virus.

1 — Epithelial cell and two lymphocytes, 3 days p.i.

2 — Alveolar macrophage, 24 hours p.i.

3 and *4* — Alveolar macrophages with pseudopodia, 24 hours p.i.

5 — Group of epithelial cells (2 elongated cells had degenerated), 6 days p.i.

6 — Polynuclear granulocytes, 3 days p.i.

7 — Ciliated epithelium cell and alveolar macrophage with pseudopodia, 3 days p.i.

Figs 8—16. Specific fluorescence of viral antigen in smears from bronchial washings, 6 days p.i. with the neurotropic strain of virus.

8 — Fluorescence in the cytoplasm of an alveolar macrophage.

9 — Epithelial cells and alveolar macrophages with specific fluorescence in the cytoplasm.

10—14 — Various types of cylindrical cells of the respiratory epithelium showing cytoplasmic fluorescence.

15—16 — A few fluorescing granules in the cytoplasm of epithelial cells.

Figs 17—21. Histological changes in the lung after inhalation of Poxvirus officinale. Haematoxylin and eosin.

17 — Some cells of the bronchiolar epithelial lining are necrotic, some regions show segmental metaplasia. The lumen is filled with oedema fluid and desquamated epithelial cells. The oedema is visible also in the peribronchial alveols; 6 days p.i. with the neurotropic strain. × 160.

18 — Widespread necrosis of the metaplastically transformed epithelial lining and also of the further layers of the bronchiolar wall. Note the irregular arrangement of epithelial cells in the metaplastic area. Dermotropic strain, 6 days p.i. × 160.

19 — Nuclear debris is visible in the completely necrotic bronchiolar mucosa. Segmental cellulization of the interalveolar septa. The necrotic changes involved also the wall of a concomittant branch of a. pulmonalis. × 63.

20 — High power view on the necrotic wall of a pulmonary artery. The advential tissue infiltrated by lymphocytes, the adjacent interalveolar septa show cellulization. × 160.

21 — Sinus of a hilar lymph node containing lymphocytes, macrophages and plasmocytes. Dermotropic strain, 6 days p.i. × 1000.