PRIMATOLOGY

Ultrastructural Stereological Analysis of the Lungs of Monkeys with Ebola Fever

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Translated from Byulleten' *Eksperimental'noi Biologii i Meditsiny*, Vol. 123, No. 2, pp. 205-208, February, 1997
Original article submitted November 18, 1995

Ebola virus replicates in mononuclear phagocytes, endotheliocytes, and, rarely, in bronchial epitheliocytes in the lungs of subcutaneously infected green monkeys, rhesus macaques, *Papio hamadryas*, and *Macaca iris*. Quantitative analysis demonstrates specific features in infection of different cells, depending on the disease duration and dose of infection.

Key Words: *Ebola virus; lung ultrastructure; monkeys*

A new outbreak of Ebola hemorrhagic fever in Zaire in May, 1995 was a new evidence of circulation of Ebola virus (EV), a most hazardous pathogen for humans [5]. Natural source and cycles of virus transmission are unknown. A filoviridae similar to EV has been isolated from green monkeys during quarantine in the USA [4,9]. Analysis of cases of infection during disease outbreaks showed that the virus is transmitted through blood and excretions [7]. Experiments with primates demonstrated the possibility of aerosol infection with Ebola fever [2]. EV replicates in the liver, adrenals, spleen, and testes of monkeys and causes destructive changes in these organs [3,6,8]. The involvement of lungs in EV infection and the possible contribution of respiratory tract cells located at the environment—organism interface to virus reproduction are practically unknown.

We studied the ultrastructure of monkey lungs at the terminal stage of EV infection.

MATERIALS AND METHODS

Ebola virus (strain Zaire) was obtained from the Byelorussian Institute of Epidemiology and Microbiology. Infecting activity of the virus in Vero cells was 5.65 lg PFU/ml and 6.77 lg LD_{50}/ml in suckling mice. Monkeys were subcutaneously infected with different dilutions of the virus. Clinically, the infection manifests itself as "all or nothing," i.e., all animals falling ill died.

For ultrastructural examination lung samples were collected from 14 green monkeys, 13 rhesus macaques, 4 crab-eating macaques, and 10 *Papio hamadryas*. The animals were narcotized with Nembutal at the terminal stage of infection. The samples were fixed in 4% paraformaldehyde, postfixed in 1% osmium tetroxide, dehydrated in ascending concentrations of ethanol and acetone, and embedded in Epon-Araldite. At least 5 blocks per animal were processed. Semithin and ultrathin sections were cut in an Ultracut ultratome; semithin sections were stained with azure II. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a JEM-100S electron microscope.

Lung samples from 4 crab-eating macaques infected with EV in doses 100 and 10 LD_{50} for this species were the object of quantitative morphological investigation. Stereological analysis was carried out as described previously [1] at magnification 10,000; for estimations a multipurpose test system of short fragments was applied directly on the screen of elec-

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0007-4888/97/0002-0178$18.00

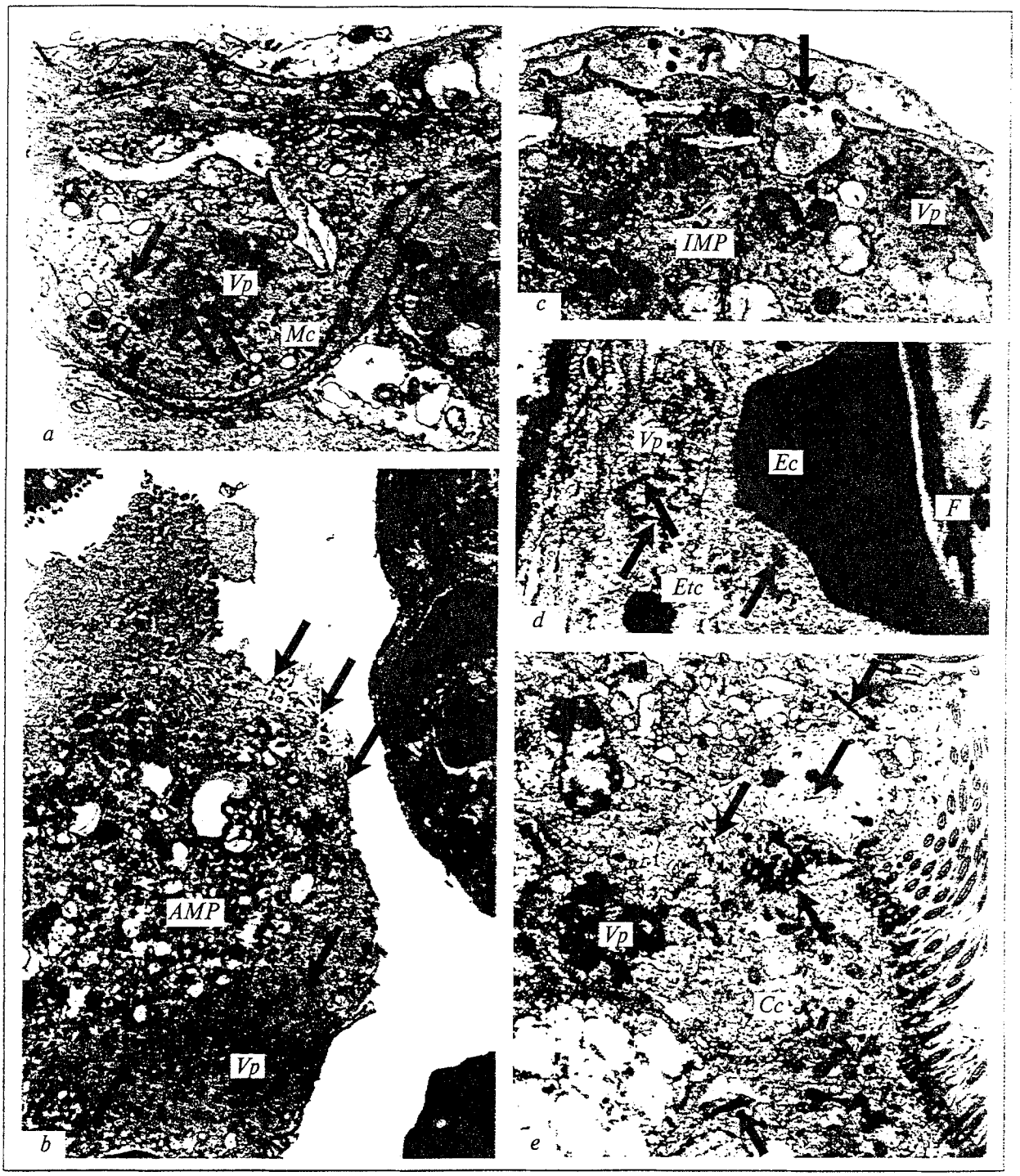

Fig. 1. Reproduction of Ebola virus (EV) in lung cells of monkeys of different species after subcutaneous infection. *a*) EV viroplasma and nucleocapsids in a monocyte of the alveolar capillary of a green monkey, day 7 postinoculation, ×7400; *b*) EV reproduction in the alveolar macrophage of rhesus macaque, day 7 postinfection, ×6200; *c*) EV reproduction in the interstitial macrophage of a rhesus macaque, day 8 postinfection, ×12,000; *d*) EV viroplasma and nucleocapsids in the alveolar capillary endotheliocyte of a crab-eating macaque, day 9 postinfection, ×14,000; *e*) EV reproduction in the bronchial ciliary cells of a green monkey, day 8 postinfection, ×6400. *Mc*) monocyte; *Etc*) endotheliocyte; *AMP*) alveolar macrophage; *IMP*) interstitial macrophage; *Ec*) erythrocyte; *F*) fibrin fibers; *Cc*) ciliary cell; *Vp*) viroplasma; nucleocapsids and virions are shown with arrows.

tron microscope. The relative volume (mm^3/cm^3) of cells, capillary bed (plasma, erythrocytes, monocytes, and neutrophils separately), fibers, and intercellular substance of the alveolar septum connective tissue and alveolar macrophages (AMP) was estimated in the respiratory tissue. The volume of alveoli was

disregarded. Infecting cells were identified by the presence of viroplasma and nucleocapsids in the cytoplasm and viral particles budding from the cell surface. The significance of the means was evaluated using Student's *t* test.

RESULTS

Examination of the lungs of EV-infected monkeys showed the involvement of lung cells in virus reproduction. In all monkeys, EV replicated in mononuclear phagocytes (MP). Virus-affected MP were located intra- and perivascularly, in the alveolar septum interstitium, and in the tracheobronchial mucosa plate (Fig. 1, *a*, *c*). In crab-eating and rhesus macaques, EV replicated in AMP (Fig. 1, *b*).

Morphological signs of EV reproduction in endotheliocytes of blood capillaries and venules were not seen in all animals (Fig. 1, *d*): in 15% of rhesus macaques, 25% of green monkeys, and 75% of crab-eating macaques and *Papio hamadryas*. Destructive changes of endotheliocytes (vacuolization and lysis of the cytoplasm and organelles) were more pronounced in green monkeys and rhesus macaques than in *Papio hamadryas* and crab-eating macaques.

The integumentary epithelium of the lung airways was dystrophically changed and desquamated at some sites. In only one green monkey did we find morphological signs of EV reproduction in the bronchial integumentary epitheliocytes (Fig. 1, *e*). The bronchial mucosa plate proper was edematous and hyperemic and contained small foci of erythrodiapedesis. Inflammatory cell infiltration was weak, with macrophages predominating over neutrophils. Lymphoid follicles in the bronchial wall of infected animals were as a rule small, without mitoses of lymphoid cells. The foci of dys- and atelectases were seen in the respiratory tract. Some alveoles were filled with exudation, AMP were scanty. Type 1 pneumocytes retained their ultrastructure, but some cells were edematous. The majority of type 2 pneumocytes were dystrophic, part of them desquamated in the alveolar lumen. Lung blood vessels and alveolar capillaries were plethoric and contained numerous neutrophils and monocytes and bundles of fibrin fibers. Hemostasis disorders played the leading role in the detected pathological changes.

The severity of destructive changes and the number of virus particles in lung tissue varied within a wide range. The greater the destructive changes, the lower the occurrence of morphological signs of viral

TABLE 1. Stereological Analysis the Lungs of Crab-Eating Macaques at the Terminal Stage of Ebola Fever

Parameter	Infecting dose, LD_{50}			
	100		10	
	Disease duration, days			
	7	9	9	12
Relative volume, mm^3/cm^3				
type I pulmonocytes	56.8±8.9	63.3±6.7	60.3±3.3	76.1±9.3
type 2 pulmonocytes	77.5±3.6	61.5±8.9	53.9±14.9	56.3±15.1
endotheliocytes	79.8±6.8	97.5±7.6	101.0±11.3	115.4±6.2
infected	12.3±3.2	17.3±3.5	n.d.	3.5±1.2
plasma capillaries	85.1±19.9	59.0±4.6*	119.1±17.5	165.3±35.0
erythrocytes	115.2±11.7	104.2±18.6	116.9±18.9	134.1±17.2
monocytes	126.3±15.1	159.9±20.5	164.1±20.7	119.6±19.9
infected	12.8±4.1	44.5±8.2	45.2±6.8	66.9±10.3
neutrophils	122.2±19.4	167.5±26.3	119.4±25.7	65.9±16.1
connective tissue macrophages	16.1±6.9	55.5±8.7*	15.3±7.1	10.2±1.7
infected	n.d.	12.0±3.4	8.1±2.7	n.d.
fibroblasts	26.9±5.1	20.9±1.0	36.6±0.8	38.5±6.2
interstitium	189.3±21.6	166.3±10.9	181.1±14.3	198.8±23.8
alveolar macrophages	104.8±16.4*	44.4±13.9	33.3±13.7	19.8±6.5
infected	n.d.	13.9±4.3	n.d.	n.d.
Total relative volume of infected cells	25.1±9.8	43.2±11.3	53.3±8.9	70.4±12.4

Note. *$p<0.05$ compared with other animals; n.d. = not determined.

reproduction. According to this regularity, the studied monkeys were arranged as follows: rhesus macaques, green monkeys, *Papio hamadryas*, and *Macaca iris*. In rhesus macaques, destructive changes were the most pronounced, while morphological signs of virus reproduction were rare. By contrast, in crab-eating macaques numerous infected cells and virus particles were found in the lungs, while alterations were negligible. Comparative titration showed that these monkeys are less sensitive to EV.

Ultrastructural stereological analysis of the lungs of crab-eating macaques helped assess the volume density of different cell structures, including those with morphological signs of virus reproduction, in the alveolar septum. Structural changes depended on the infecting dose (Table 1). In animals infected with 100 LD_{50}, the volume density of infected endotheliocytes was 15.4% on day 7 and 17.7% on day 9 of infection, while after 10 LD_{50} no infected endotheliocytes were detected on day 9 and only 3% of them carried EV on day 12 of experiment ($p<0.05$). On the other hand, 10.1% EV-positive monocytes were identified in animals infected with 100 LD_{50} on day 7 of infection, which was significantly lower in comparison with other animals ($p<0.05$) and virtually the same for different infecting doses (27.8 and 27.5%) on day 9 of experiment. On day 12 after infection with 10 LD_{50}, this parameter was 55.9%.

The volume density of interstitial and alveolar macrophages was higher in monkeys infected in a dose of 100 LD_{50}. Presumably, these changes are due to the fact that even after infection with a higher dose, the proportion of infected MP was still low, and there were cells capable of differentiating in resident macrophages in the population of intact monocytes.

Analysis of quantitative data indicates that the relative proportion of infected monocytes markedly increased during the disease and virtually did not depend on the infecting dose. The increment of the relative volume of infected endotheliocytes in the course of infection was slower and strongly depended on the infecting dose.

Hence, mononuclear phagocytes, endotheliocytes, and, extremely rarely, bronchial epitheliocytes participate in EV reproduction in the lungs of monkeys. Taking into consideration the contribution of the respiratory tract cells (AMP and bronchial epitheliocytes) to virus replication, we have hypothesized that Ebola virus is transmitted by inhalation of infected droplets.

The study was supported by the International Research Foundation and Russian Government (grant No. JFL 100).

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