VIROLOGY

Study of the Pathogenesis of Ebola Fever in Laboratory Animals with Different Sensitivity to This Virus

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 132, No. 12, pp. 664-668, December, 2001
Original article submitted June 7, 2000

Pathophysiological parameters were compared in animals with different sensitivity to Ebola virus infected with this virus. Analysis of the results showed the differences in immune reactions underlying the difference between Ebola-sensitive and Ebola-resistant animals. No neutrophil activation in response to Ebola virus injection was noted in Ebola-sensitive animal. Phagocytic activity of neutrophils in these animals inversely correlated with animal sensitivity to Ebola virus. Animal susceptibility to Ebola virus directly correlated with the decrease in the number of circulating T and B cells. We conclude that the immune system plays the key role in animal susceptibility and resistance to Ebola virus.

Key Words: *Ebola virus; pathogenesis; sensitivity*

By morphological, genetical, and antigenic signs Ebola virus (EV) is referred to *Filoviridae*. The prototype virus of this family is Marburg virus isolated previously. The pathogenesis of Ebola fever is complex and involves many organs. Despite many-year studies of the pathogenesis of this infection, there is no universal concept on triggering mechanisms of this fulminate disease [14].

Most animals (*e. g.* rabbits) are resistant to EV and develop no clinical signs of the disease after inoculation of the virus. Some animals (guinea pigs) are weakly susceptible to EV: virus inoculation in these animals induce fever and body weight loss, but the disease eventuates in recovery, unlike highly susceptible animals (primates) in which the disease rapidly eventuates in death. We investigated a methodological approach to detection of pathophysiological processes essential for the pathogenesis of Ebola fever by comparing the dynamics of immune, hematological, and biochemical parameters and parameters of blood clotting in animals differing by sensitivity to EV in response to inoculation with the virus.

The term "sensitivity" was used to denote cases of weak and slight but clinically manifest susceptibility, as we consider the mechanisms of animal sensitivity to EV the most important in studies of the pathogenesis of Ebola fever, to say nothing about carriership and asymptomatic infection. The term "resistance" was used to denote the absence of susceptibility, and resistance, carriership, and asymptomatic infection were called "insensitivity".

MATERIALS AND METHODS

Experiments were carried out on rabbits and guinea pigs from vivarium of Vektor Research Center, kept under standard vivarium conditions. The animals were infected with EV subtype Zaire. Two strains were used. Strain Zaire 1976 is not virulent for rabbits and causes a nonlethal infection in guinea pigs; it was obtained from the National Collection of Virology Center

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0007-4888/01/1326-1182$25.00

(Institute of Microbiology, Ministry of Health of Russian Federation) the virus was produced in Vero cell culture and purified in sucrose density gradient. Strain 8 ms causing a lethal infection in guinea pigs was obtained by adaptation of Zaire 1976 strain to guinea pigs by serial passages.

In experimental series I, 2 rabbits (1.5-2.0 kg) and 10 guinea pigs (200-300 g) were intramuscularly inoculated 3 times with 16-day intervals with the virus preparation (100 ml for rabbits and 30 ml for guinea pigs) containing EV Zaire 1976 strain (6-8×10^8 burst-forming units (BFU)/ml). The animals were observed for up to 51 days.

In series II, guinea pigs (150-180 g) were inoculated with the same strain in a single dose of 10^4 BFU/ml or with strain 8 ms (10^4 LD_{50}/ml) intraperitoneally, the period of observation being 20 and 9 days, respectively.

Blood was collected before inoculation (baseline values) and then twice a week during the entire period of observation. Blood was collected from each rabbit (from the ear vein) and from several guinea pigs per group (from the heart). All painful manipulations were carried out under analgesia.

For immunological studies, blood was collected into tubes with heparin (100 IU/ml), for biochemical studies into dry tubes for preparing serum, and for coagulation tests into silicone tubes with 1% EDTA (1:9). For separating the plasma, blood was centrifuged for 10 min at 1500 rpm. For hematological studies both heparin-treated and EDTA-treated blood was used.

Circulating T and B cells were counted and neutrophil phagocytic activity were evaluated as described previously [3]. Titers of total antibodies to EV were evaluated as described elsewhere [7]. The level of circulating immune complexes was evaluated [2].

Activities of aminotransferases, levels of malonic dialdehyde (MDA), β-lipoproteins, and urea were measured as described elsewhere [5].

The prothrombin index was estimated and ethanol test was performed [2], fibrinogen content was evaluated by the gravimetric method. Parameters of platelet hemostasis were evaluated as described previously [9].

The total counts of blood leukocytes and erythrocytes were evaluated by routine clinical methods. For differential leukocyte count thin smears of he-

TABLE 1. Pathophysiological Reactions in Animals Inoculated with EV

Parameters	Rabbits; strain Zaire 1976	Guinea pigs	
		strain Zaire 1976	strain 8 ms
Blood cell composition			
Increased neutrophil count	—	±	++*
Increased percentage of eosinophils	N. d.	±	+
Decreased percentage of monocytes	—	±	++
Young granulocyte forms	N. d.	±	++
Increased percentage of young platelets	N. d.	±	++
Biochemical parameters			
Increased aspartate-alanine aminotransferase activities	—/—	+/+	++/+
Decreased/increased MDA content	—/—	+/—	++/++
Increased urea/β-lipoprotein content	—/—	±/—	++/++
Parameters of blood clotting system			
Increased prothrombin index	+	+	+
Positive ethanol test	+	+	+
Increased fibrinogen content	N. d.	±	++
Increased/decreased platelet count	N. d.	++/+	++/++
Immune status			
Activation of neutrophil phagocytosis	++	±	—
Increased count of T/B lymphocytes	+/+	±/±	—/—
Increased level of circulating immune complexes	N. d.	+	—
Antibodies to EV	++	±	—

Note. "—" no changes; "±" minor changes; "+" moderate changes, "++" pronounced changes. N. d. no data. *Vacuolation of the neutrophil cytoplasm, toxic granulation during the pre- and terminal stages of infection.

parin-treated blood were fixed in 96% ethanol for 10 min and stained after Nocht.

The results were statistically processed using Student's *t* test and dispersion analysis methods using the protocol of the bifractional experiment with repeats.

RESULTS

Both susceptible and resistant animals inoculated with EV demonstrated increased prothrombin index and positive ethanol test (Table 1), which attested to an imbalance in the blood clotting system. However in animals resistant to EV the clotting reactions had limiting components, which allowed normalization of the hemostasis parameters, while the susceptible animals had a source "feeding" the coagulation cascade eventually leading to development of the lethal coagulopathic syndrome.

Immune complexes were suggested as a factor provoking blood clotting disorders in hemorrhagic fevers [12]. We believe that the cytotoxic effects of immune complexes consisting of EV antigen and anti-EB antibodies promote coagulation cascade in Ebola fever. We detected elimination of circulating immune complexes in guinea pigs infected with EV strain 8 ms (Fig. 1, *a*), but not in guinea pigs injected with EV strain Zaire 1976, in which the circulating immune complexes did not disappear. Moreover, guinea pigs infected with EV strain 8 ms produced autoantibodies reacting, within immune complexes, with endothelial cells.

These findings together with accumulation of MDA (indicating extensive membrane lesions), appearance of juvenile forms of platelets and granulocytes, increased percentage of eosinophils (Table 1) [3,9] suggest that the cytotoxic effects of immune complexes consisting of EV antigen, anti-EV antibodies, and autoantibodies promote the development of coagulation cascade in Ebola fever. Deposition of immune complexes in the vascular intima and tissues provokes the release of toxic products from damaged tissue which, as blood

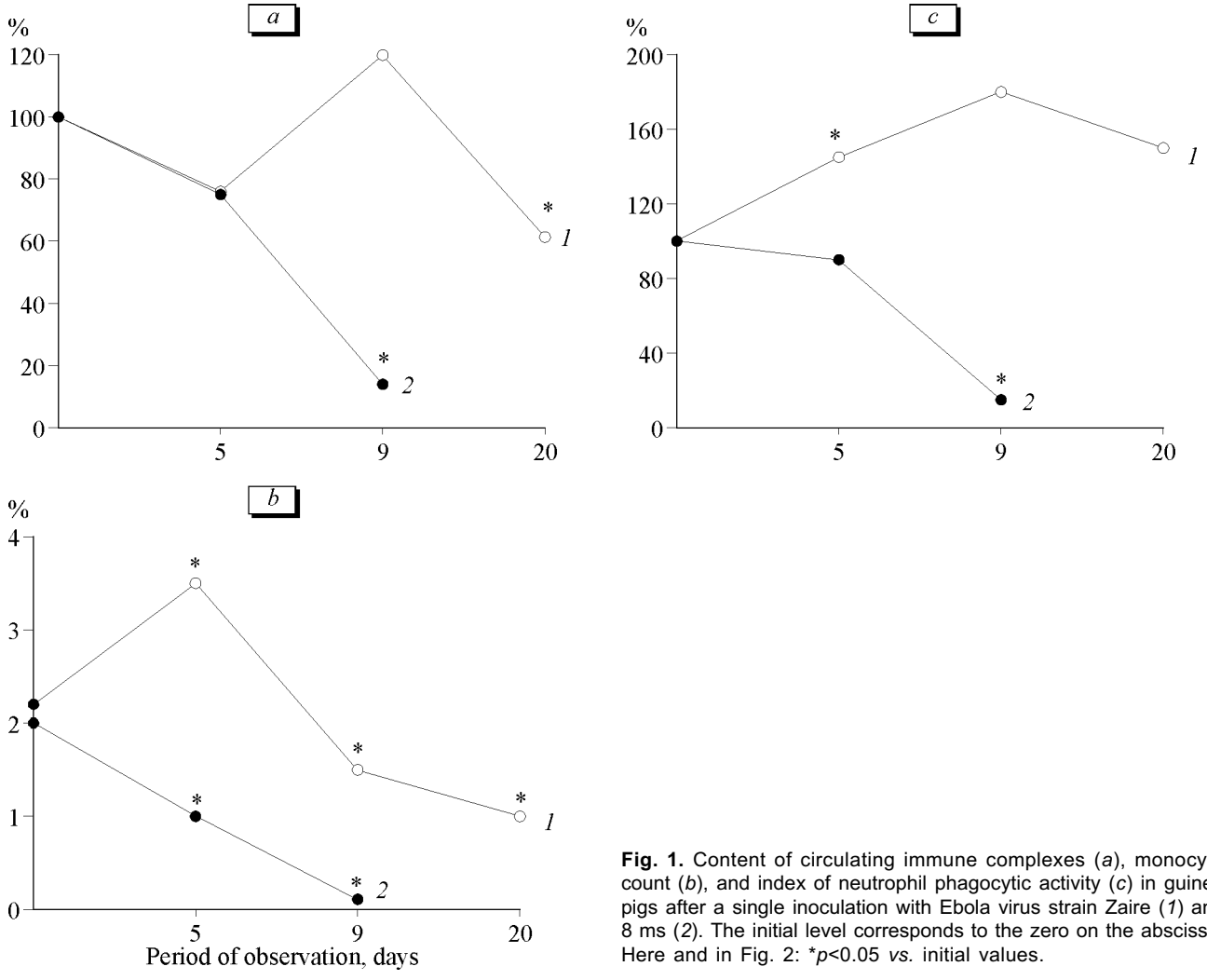

Fig. 1. Content of circulating immune complexes (*a*), monocyte count (*b*), and index of neutrophil phagocytic activity (*c*) in guinea pigs after a single inoculation with Ebola virus strain Zaire (*1*) and 8 ms (*2*). The initial level corresponds to the zero on the abscissa. Here and in Fig. 2: *$p<0.05$ *vs.* initial values.

clotting system activators, become the sources maintaining the development of the blood clotting cascade.

Elimination of circulating immune complexes from the bloodflow of guinea pigs infected with EV strain 8 ms can be attributed to their absorption by activated macrophages, which are known to play a key role in elimination of damaged cells, microorganisms, and immune complexes. However filovirus infection leads to generalized destruction of macrophages in guinea pigs and monkeys [8]. Moreover, we observed disappearance of monocyte sprecursors of tissue macrophages, from the blood of guinea pigs infected with EV strain 8 ms (Fig. 1, *b*). This indirectly confirms macrophage destruction during infection, as accelerated migration of monocytes from the circulation for differentiation into tissue macrophages is caused by generalized death of these cells. Hence, active involvement of macrophages in elimination of immune complexes is hardly probable. Neutrophils also can phagocytize the immune complexes. However, decreased phagocytic activity of neutrophils in guinea pigs infected with EV strain 8 ms (to 15% of the initial level) during terminal stage of the infection (Fig. 1, *c*) and simultaneous decrease in the level of circulating immune complexes (to 14% of the initial level) suggest that the disappearance of immune complexes from the blood was due to their precipitation in tissues.

Analysis of the results showed parallel changes in the levels of circulating immune complexes, monocyte count, and phagocytic activity of neutrophils in guinea pigs infected with EV strain 8 ms, which attests to a direct relationship between these parameters. The progressive increase of the neutrophil phagocytic activity in guinea pigs inoculated with EV strain Zaire in comparison with those infected with strain 8 ms indicates that neutrophil phagocytic activity in animals susceptible to EV is notably lower than in weakly sensitive animals. However in resistant animals the phagocytic activity of neutrophils is much higher than in animals weakly susceptible to EV (Fig. 2). Hence, the phagocytic activity of neutrophils inversely correlates with animal sensitivity to EV.

The existence of a factor blocking neutrophil activation and the relationship between this phenomenon and sensitivity to EV are obvious. Results of comparative molecular-biological study of genomes of Zaire 1976 and 8 ms strains suggest that this factor is located in EV protein vp24. Functional activity of neutrophils in Ebola fever was not studied in detail, but numerous functions of neutrophils in immunogenesis prompt the study of the role of these cells, along with other immune cells, in the pathogenesis of Ebola fever.

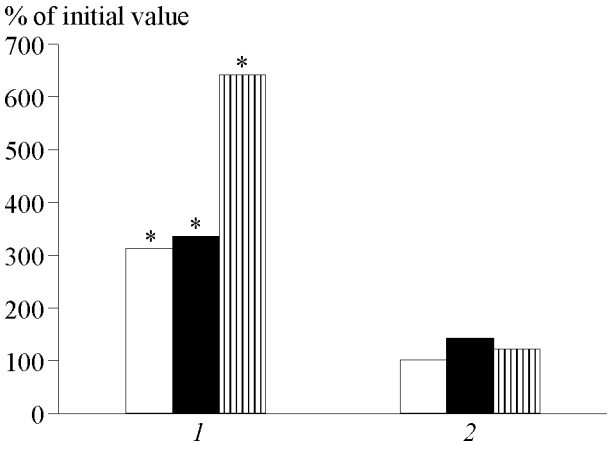

Fig. 2. Neutrophil phagocytic activity in rabbits (*1*) and guinea pigs (*2*) receiving three injections of Ebola virus strain Zaire. Light bars: 1st injection; dark bars: 2nd injection; cross-hatched bars: 3rd injection.

Hence, animals with different susceptibility to EV showed oppositely directed changes in immunological, hematological, biochemical, and blood-clotting parameters. Immunity was activated in resistant animals and suppressed in susceptible; *vice versa*, no activation of the above complex of parameters was observed in resistant animals and their marked elevation in susceptible animals. Essential differences in the reactivity of immunological parameters, particularly the neutrophil phagocytic activity and T cell count, indicate the key role of the immune system in animal susceptibility to EV.

Summing up these results, we should emphasize that most studies were carried out only on animals sensitive to the studied agents and on animals with polar or opposite sensitivity to the infectious agent [4]. This approach helped us differentiate the factors responsible for the disease outcome. We suggest studies on animals ranked by sensitivity to the agent as a methodological approach to studies of Ebola fever. This approach allows more effective detection of factors critical for the pathogenesis of this infection, which extends the modern concepts on its pathogenesis.

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