VIROLOGY

Effect of Ebola Virus Reinoculations on the Time Course of Immunological Parameters

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Three inoculations of live and inactivated Ebola virus to susceptible (guinea pigs) and resistant (rabbits) animals helped reveal some regularities in the immunity reactions which have not been observed after a single inoculation: an increase in the count of T suppressors both in rabbits and guinea pigs after reinoculations of the virus preparations and the resistance of T lymphocytes in rabbits and their suppression in guinea pigs. Similar immunogenicity of live and inactivated Ebola virus for guinea pigs and a higher immunogenicity of live virus for rabbits deserves attention.

Key Words: *Ebola virus; immunity; vaccination; immunogenicity; T suppressors*

Ebola virus (EV) causes severe hemorrhagic fever with high mortality (up to 88%) in humans. Infection of guinea pigs (conditionally susceptible to EV) with EV leads to virus replication and generally to a nonlethal disease, while EV inoculation in rabbits (resistant to EV) causes no clinical symptoms of disease or signs of EV replication. Apparently, different immunological reactions develop in these animals after EV inoculation. Study of these differences will provide new data on the immunopathogenesis of Ebola fever and promote vaccine development.

Published data on protective immunity against EV are contradictory [4,12,13]. Multiple vaccinations are recommended in some infectious diseases. We deemed it interesting to study the time course of some immunological parameters in rabbits and guinea pigs after reinoculations of live and inactivated EV. Reinoculations of live EV to guinea pigs were regarded as immunization with attenuated virus. The criteria of comparison were the counts of T and B lymphocytes in peripheral blood, their proliferative activity in response to stimulation with concanavalin A, and the count of T-suppressors (TS).

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MATERIALS AND METHODS

EV subtype Zair, strain Zair (nEV) was obtained from the Virology Center at the Institute of Microbiology, Ministry of Defense. The virus was grown in Vero cells and purified in sucrose gradient [10]. The amount of protein in purified preparation was 1 mg/ml at ele-ctrophoretic purity 95%. The virus was inactivated by heating for 1 h at 58°C (inEV). The absence of residual virulence was verified on newborn albino mice.

Rabbits (1.5-2 kg) and guinea pigs (200-300 g) from the vivarium of the Vektor Research Center were maintained on standard rations. The rabbits were divided into 2 groups, 2 animals each, guinea pigs into 2 groups, 10 animals each.

To animals of the first groups, nEV was injected intramuscularly three times at 16-day intervals in doses of 100 and 30 μl, respectively (the biological titer was $6\text{-}8\times10^8$ plaque-forming units/ml). Animals of groups 2 were inoculated with equivalent amounts of inEV at the same intervals.

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Blood was collected once before the experiment (baseline levels) and then twice a week throughout the entire experiment (up to 51 days). In rabbits, the blood was collected from the ear veins from each animal separately, from guinea pigs the blood was collected from the heart from several animals of each group at random. All painful procedures were carried out under anesthesia.

The proliferative activity (PA) of lymphocytes stimulated with concanavalin A was evaluated in whole heparin-treated blood in the lymphocyte blastogenesis test [6] in our modification [11]. The lymphocyte PA index was calculated from the formula: $I=(M-K/Mi-Ki)\times100\%$, where Ki and Mi are the numbers of blasts in control and experimental samples in the initial portion of blood and K and M are those after inoculation of the virus.

T and B lymphocytes were counted in the E and EAC rosette formation tests [7]. In guinea pigs, the T lymphocyte population was analyzed using rabbit erythrocytes [14] and B population using the same erythrocytes treated with the complement. The results of estimation of the number of rosette-forming cells (per 200 lymphocytes) were transferred into absolute values with regard for the total count of leukocytes and percentage of blood lymphocytes. The indices of T and B rosette formation were calculated as the ratio of the number of rosette-forming cells in experimental and initial portions of the blood multiplied by 100%.

TS were counted by evaluating their theophylline sensitivity as described previously [2]. Analysis of correlations with the use of monoclonal antibodies [5,8] confirmed the correctness of theophylline use for counting TS lymphocytes, which is important for manipulations with animal lymphocytes.

The data on each animal on the days of blood collection after each inoculation with the virus were summed up and the mean values for the periods between inoculation were calculated. The significance of differences was estimated using Student's t test.

RESULTS

The rabbits developed no temperature reaction to inoculation with nEV or inEV. In guinea pigs rectal temperature increased by 0.5-1°C after the first inoculation with nEV, and after reinoculations only some animals developed fever. Inoculation with inEV did not lead to fever in any of the guinea pigs.

After each inoculation with nEV and inEV, the count of T lymphocytes increased in rabbits and decreased in guinea pigs (Fig. 1, *a*).

In rabbits, the count of B lymphocytes increased almost twofold after the first inoculation with nEV *vs.* the initial values (Fig. 1, *b*). Reinoculations of rabbits with nEV led to a progressive decrease in the number of circulating B lymphocytes to 40% of the baseline level. The first inoculation of inEV caused a negligible decrease in the count of B lymphocytes in rabbits, and reinoculation of this preparation led to a pronounced decrease in B cell count. In guinea pigs, the pattern of changes in the counts of B lymphocytes in response to nEV and inEV (Fig. 1, *b*) was similar to that in rabbits after inEV.

Proliferative activity of rabbit lymphocytes after nEV was two times higher than after inEV, which caused a slight stable increase in this parameter (Fig. 2, *a*). In guinea pigs, lymphocytic PA decreased after the first inoculation with nEV and inEV in comparison with baseline level, while subsequent inoculations of both preparations progressively increased PA to 200% of the initial value (Fig. 2, *a*).

The time course of TS changes was similar in rabbits and guinea pigs inoculated with nEV and inEV (Fig. 2, *b*). Reinoculations led to an increase in the percentage of TS: up to 90% in guinea pigs and 76% in rabbits after the third inoculation.

The time course of changes in some parameters was opposite in the rabbits and guinea pigs inoculated with VE: lymphocyte PA and T lymphocyte count increased in rabbits, while in guinea pigs the count of T cells persistently decreased and the time course of PA was different. Reinoculations of EV to susceptible and resistant animals helped disclose some regularities in the development of immunological reactions.

Multiple inoculations of EV to guinea pigs led to a decrease in the number of circulating T and B lymphocytes. Unlike the first inoculation, the third one caused a marked increase in the TS percentage, which was 80-90% of the total T lymphocyte population after both nEV and inEV. High PA of the total lymphocyte pool (with predominance of TS) in guinea pigs after the third inoculation of EV suggests that EV progressively activates TS. This was observed in both EV-susceptible and resistant animals.

A progressive decrease in the count of B lymphocytes in rabbits and guinea pigs after EV reinoculations is due to increased count of TS lymphocytes. The formation of suppressors is believed to depend on the intensity of antigenic stimulation [1]. The higher the stimulation (higher antigen dose or frequent antigen challenge), the more intense the suppressor formation [15]. TS lymphocytes, which affect humoral and cell-mediated immunity, are formed in different quantities. In our experiment, three inoculations of EV at 16-day intervals can be regarded as a potent exposure to antigen, intensifying the formation of suppressors. After the first inoculation that sensitized the organism and primed the immune cells, reinoculations caused a progressive increase in the counts of TS lymphocytes,

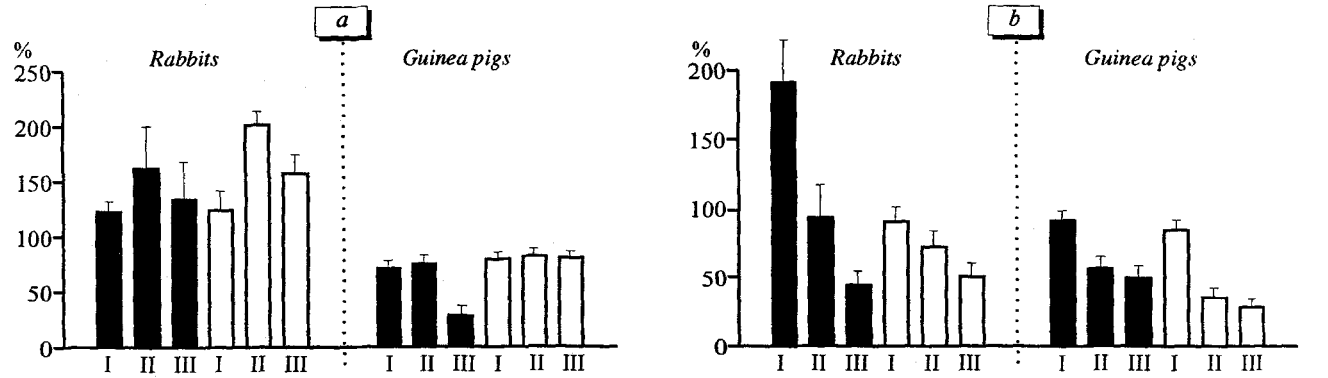

Fig. 1. Time course of the index of T (*a*) and B rosette formation (*b*) in rabbits and guinea pigs inoculated three times with live (dark bars) and inactivated (light bars) Ebola virus. Here and in Fig. 2: Roman figures show the order of inoculations. The initial values of the indices are taken for 100%.

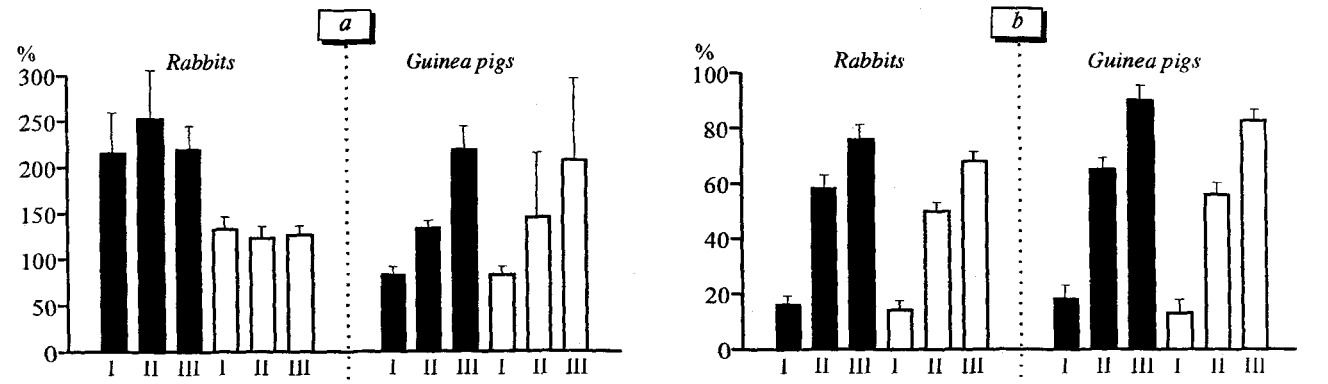

Fig. 2. Time course of the proliferative activity index (*a*) and lymphocyte T suppressors (*b*) in rabbits and guinea pigs inoculated three times with live (dark bars) and inactivated (light bars) Ebola virus.

among which the humoral response suppressors predominated in the rabbits, while in guinea pigs the effects of TS lymphocytes on the T and B immunity was the same.

Multiple inoculations with EV helped define the T/B immunity ratio in rabbits, consisting in suppression of B lymphocytes and in stability of T lymphocytes after reinoculations. The resistance of T lymphocytes in these animals (positive changes in their absolute counts in peripheral blood) after reinoculations with EV and the absence of this resistance in B lymphocytes suggests that the resistance of rabbits to EV is largely due to cell-mediated, but nor humoral immunity.

The type and intensity of immune response of guinea pigs to nEV and inEV is virtually the same, as evidenced by the immunological values. It means that thermoinactivation of EV leads to the loss of its infectiveness for guinea pigs but does not modify the immunological reactions of the organism, i.e., the immunomodulating activity of EV is due to its proteins. This fact can be regarded as positive because similar immune responses to inEV and nEV implies the probability of developing a specific prophylactic agent on the basis of inEV.

Unlike in guinea pigs, in rabbits the immune responses to nEV and inEV were different. The first inoculation of nEV caused a twofold increase in the B lymphocyte count, while inoculation with inEV did not change it. The lymphocyte PA in these animals in response to nEV inoculation was higher than in response to inEV. These facts demonstrate higher immunogenicity of EV in comparison with inEV. Immunization with nEV more effectively stimulated the production of virus-neutralizing antibodies in animals resistant to EV, which proves higher immunogenicity of nEV in comparison with inEV for animals unsusceptible to EV [12]. It may be due to partial destruction of antigenic determinants during the virus inactivation.

The difference in the quality of immunological reactions consists in the lack of T-cell suppression in animals resistant to EV, suggesting a relationship between the resistance to EV and the activity of T lymphocytes. Previously we observed pronounced activation of the neutrophil phagocytic capacity in rabbits and its decrease in guinea pigs [3]. Taken together with the data obtained in this study, these results indicate that cellular mechanisms of immunity, both specific and nonspecific, determine the resistance of an organism to EV.

Ebola virus suppresses T-cellular immunity in guinea pigs, this suppression being the most pronounced after reinoculations of nEV and inEV; therefore, multiple vaccinations with inEV seem to be unfit for effective protection of animals susceptible to EV. Hence, the most acceptable method for immunization is a single inoculation with inEV in parallel with immunostimulation. Previous data on 80% protective effect of double immunization of Papio hamadrias with inEV in complete Freund's adjuvant, which was followed by infection with EV, demonstrate the efficacy of immunostimulation [4]. The immunopotentiating effect of complete Freund's adjuvant, increasing the activity of T cells (specifically, the function of T helpers), is so high that it is not allowed to be used in humans [9]. However, testing of more "mild" immunostimulants and various immunizing doses of inEV may lead to a combination that will protect from subsequent infection with EV.

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