ISSN 0026-8933, Molecular Biology, 2021, Vol. 55, No. 3, pp. 413–423. © Pleiades Publishing, Inc., 2021.
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MOLECULAR CELL BIOLOGY

UDC 578.282

Preventive Vaccination with Mesenchymal Stem Cells Protects Mice from Lethal Infection Caused by Herpes Simplex Virus 1

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Received August 13, 2020; revised September 10, 2020; accepted September 10, 2020

Abstract—Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) infect almost all organs and tissues, cause genital herpes—the most common sexually transmitted disease—disorders of the central nervous system (CNS), and lead to severe complications in children. Despite the available drugs, the incidence of HSV-1/2 continues to rise. None of the prophylactic vaccine candidates have shown a protective effect in trials nor approval for use in clinical practice. We have investigated the protective properties of mesenchymal stem cells (MSC) isolated from the bone marrow of mice. A comparative analysis of the protective response to the introduction of primary and modified MSCs (mMSC) was carried out using the plasmid containing gene of the HSV and an inactivated virus in a model of lethal HSV-1 infection in mice. mMSCs were obtained by transfection of the *Us6* gene encoding glycoprotein D (gD) of the HSV, the plasmid contained the same gene. After twofold immunization with primary MSCs, the formation of antibodies interacting with the viral antigen (according to enzyme immunoassay data) and neutralizing the infectious activity of HSV-1 in the reaction of biological neutralization was observed in the peripheral blood of mice. In addition, the introduction of primary MSCs induced the production of interferon gamma (INF-γ) which is detected in the peripheral blood of mice. After infection with HSV-1, the immunized mice showed significantly increased titers of virus-specific antibodies, an increased level of IFNγ, and were completely protected from lethal HSV-1 infection. The protective effect of the other three immunogens was lower and did not exceed 50–65%. Considering the wide availability of MSCs, the proven safety of intravenous administration, and the results obtained in this work on the ability to induce innate, adaptive and protective immunity to HSV-1, MSCs can be considered a promising basis for the development of new cellular vaccines for the prevention of herpesvirus and other viral infections.

Keywords: mesenchymal stem cells, transfection, genetically modified mesenchymal stem cells, recombinant DNA, DNA immunization, herpes simplex virus 1, protective activity

DOI: 10.1134/S0026893321020242

INTRODUCTION

Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) are ubiquitous human pathogens: 3.7 billion are infected with HSV-1 and 417 million are infected with HSV-2 [1–3]. According to the WHO [4], genital herpes is recognized as the most common sexually transmitted infection and it has been proven that HSV infection increases the risk of HIV infection [5]. Both viruses cause neonatal herpes [6]. HSV-1 is the leading etiological agent of infectious blindness; 1 million new cases and 9 million recurrent episodes of HSV eye infection are registered in the world every year [7]. Currently, many drugs are available for the treatment of herpes infection, but the incidence of HSV continues to increase [8]. In this regard, the development of an effective prophylactic vaccine against HSV-1/2 is recognized by WHO as a public health priority area (http://www.who.int/immunization/global_vaccine_action_plan/GVAP_doc_2011_2020/en/) [9, 10].

Currently, a number of vaccines against herpesvirus infections have been developed, these are based on recombinant DNA and HSV proteins, peptides, live attenuated viruses, and inactivated whole virion preparations [11–13]. However, most of the candidate vaccines have not been shown to have a protective effect in clinical trials [14], and so far none of the prophylactic vaccines have received approval for use in medicine.

Obviously, new approaches for the creation of herpes vaccines are needed, one of which was studied in this work. This approach is based on the use of mesenchymal stem cells (MSC), which have been successfully used in various fields of regenerative medicine. Approaches are being developed to obtain genetically

Abbreviations: HSV-1, herpes simplex virus 1; iHSV, inactivated whole HSV-1; DNAgD, recombinant DNA containing gD protein gene of HSV; PSI, proliferation stimulation index; LD_{50}, lethal dose, 50%; MSCs, mesenchymal stem cells; mMSCs, genetically modified MSCs; MSCgD, mSCs that stably produce the gD protein of HSV; RBT, reaction of blast transformation.

modified MSCs (mMSCs) that express target genes and can be used for cell therapy of cancer, autoimmune, neurodegenerative, and infectious diseases [15–20]. Based on these data, one can make a conclusion about the plasticity of immunomodulation of MSCs, which can have both suppressive and immunostimulating effects. There are now publications showing the fundamental possibility of using MSCs and mMSCs as innovative vaccines that enhance immune responses against HIV [21] and the hepatitis C virus [22].

The objective of this work was to comparatively analyze the protective immune response to the introduction of prophylactic candidate vaccines based on MSCs, a DNA plasmid, and an inactivated virus against lethal HSV-1 infection in mice.

MATERIALS AND METHODS

Animals. DBA/2J (H-2^d) mice (females, 6–8 weeks old) were obtained from the Stolbovaya Central Nursery for Laboratory Animals (Moscow Oblast).

Cell cultures. The primary culture of MSCs was obtained from the red bone marrow of mice, as described previously [23]. We have shown [22, 23] that in terms of adhesive ability, surface markers, and differentiation potential, these cells meet the criteria for determination as MSCs by the International Society for Cellular Therapy [24].

Cultivation of MSCs was carried out in DMEM medium containing 10% fetal bovine serum (FBS; Gibco, United States), 10 μg/mL insulin, 5.5 μg/mL transferrin, 6.7 ng/mL selenite, 10 ng/mL fibroblast growth factor, 2 mM L-glutamine, and 50 μg/mL gentamicin in an atmosphere of 5% CO_2 at 37°C.

The culture of the African green monkey kidney (Vero) cells was obtained from the collection of cell cultures of the Gamaleya National Research Centre for Epidemiology and Microbiology and cultured under the same conditions using Eagle's minimal essential medium (MEM) supplied with 10% FBS (Biosera, France), 2 mM L-glutamine, and 50 μg/mL gentamicin. Culture media, sera and components for the cultivation of cell cultures, unless indicated otherwise, were purchased from PanEko (Russia).

Virus. HSV-1, obtained from the State Virus Collection of the Gamaleya National Research Centre for Epidemiology and Microbiology, was propagated in Vero cells using standard cultivation methods. The multiplicity of viral infection was determined by a modified in vitro plaque assay. A series of dilutions of virus samples were prepared and introduced into 96-well plates with a monolayer of cells, and incubated at 37°C for 7 days in the presence of 5% CO_2. The foci of infected cells (plaques) were identified and counted using a Primovert inverted microscope (Zeiss, Germany). The virus titer was determined using the formula: $A = ab/V$, where A is the number of plaque-forming units per 1 cell (PFU/cell); a—average number of plaques per well; b—virus dilution; V—volume of vaccinated material introduced.

Determination of the lethal dose, 50% (LD_{50}) of HSV-1 in vivo was carried out by infecting animals intraperitoneally with 10-fold serial dilutions of the virus. The death of animals was recorded within 14 days of infection. The calculation of the lethal dose of HSV-1 was carried out according to the formula: $LD_{50} = 1 \times 10^{X + [(C1 - 50)/(C1 - C2)]}$, where X is the reciprocal of the virus dilution at which 50% death of mice was observed; C1 is the proportion of mice (%) that died at the maximum dilution, which gave a lethality above 50%; and C2 is the proportion of mice (%) that died at the minimum dilution, which gave a lethality below 50%.

Obtaining an inactivated HSV-1. Vero cells were planted in 175 cm^2 culture flasks and, upon reaching a monolayer, were infected with HSV-1. After the manifestation of the maximum virus-specific cytopathic effect (CPE), 1 cycle of freezing-thawing was performed. The virus-containing culture liquid was centrifuged (Jouane, France) at 10000 rpm for 10 min to remove cell debris, and the virus was precipitated from the resulting supernatant by ultracentrifugation at 30000 rpm for 1 h at 4°C (SW32 rotor; Beckman, United States). The pellet was resuspended in 1 mL of 10 mM Tris-HCl, pH7.4, and left overnight at room temperature. Then 2 mL of 60% sucrose, 10 mL of 15% sucrose, and vaccinated suspension were sequentially layered into a centrifuge flask. Ultracentrifugation was carried out under the conditions described above. Purified HSV-1 was inactivated at 56°C for 30 min. Inactivated virus (iHSV) was used to immunize animals and as an antigen for enzyme-linked immunosorbent assay (ELISA).

Plasmid. We used the DNA construct pcDNAgD (DNAgD), obtained on the basis of the commercial plasmid pcDNA-3.1(+) (Invitrogen, United States), into which the *Us6* gene encoding the gD protein of HSV and the marker gene *neo* (neomycin phosphotransferase) were inserted. To develop the construct, *Escherichia coli* JM109 cells were used, which were grown in a nutrient medium (LB broth, LB agar, and SOB broth) consisting of bactotrypton, yeast extract, and bactoagar (Difco, United States). The plasmid was isolated using a commercial QIAGEN Plasmid Purification Maxi Kit (QIAGEN, United States) according to the manufacturer's instructions.

Transfection of MSC cultures. To obtain genetically modified MSCs (mMSCs) expressing the *Us6* gene of HSV, we used a primary culture of MSCs at the fourth passage. The cells were transfected with Lipofectamine 3000 (Thermo Fisher Scientific, United States) according to the manufacturer's recommendations. The MSCs were seeded in a 24-well plate at a concentration of 2×10^4 cells/mL in DMEM growth medium without antibiotics. After 24 h, a complex of DNAgD and Lipofectamine 3000 was intro-

duced into the cells at a ratio of 1 : 2. The selection of transformants was carried out in a culture medium containing geneticin G-418 (Invitrogene). After 5 and 14 days, the expression of the *Us6* gene was analyzed in the cells obtained as a result of selection. The cells were fixed with chilled methanol and treated with monoclonal antibodies against the gD protein of HSV (ab6507; Abcam, UK) for 1 h at 37°C. Unbound antibodies were washed with phosphate-buffered saline (PBS), then anti-mouse antibodies conjugated with horseradish peroxidase (anti-mouse PX, PO260; Dako, Denmark) were layered, and incubated for 30 min at 37°C. The cells were washed with PBS followed by addition of solution of 3,3'-diaminobenzidine at a concentration of 0.05 mg/ml in 0.05 M Tris-HCl buffer (pH 7.4) supplemented with 3% hydrogen peroxide. The reaction was stopped after 10 min by adding distilled water to the wells. The results were evaluated using an AxioScopeA1 microscope (Zeiss, Germany). The number of stained cells containing the gD protein of HSV was counted and presented as a percentage of the total number of cells in the population.

To study the production of cytokines secreted by mMSCs, the culture fluid was taken during 14 days of MSC selection and the cytokine concentration in it was determined as described below.

Immunization. Animals divided into 5 groups were injected with primary MSCs at a concentration of 5×10^5 cells/mouse intravenously (MSCs; group 1, $n = 15$); with mMSCs (modified MSCs expressing the *Us6* gene of HSV), at a concentration of 5×10^5 cells/mouse intravenously (MSCgD; group 2, $n = 10$); with recombinant DNA containing the *Us6* gene of HSV, at a concentration of 100 μg/mouse intramuscularly (DNAgD; group 3, $n = 20$); and with inactivated virus, at a concentration of 100 μg/mouse intravenously (iHSV; group 4, $n = 10$). Group 5 ($n = 20$) was used as a control, where the mice were injected intravenously with normal saline solution. Immunization was performed twice with an interval of 14 days.

Infection. Mice of groups 1–5 were infected with HSV-1 (15 LD_{50} intraperitoneally) 14 days after the last immunization. The animals were observed daily for 14 days. The survival rate of animals was calculated as a percentage (%).

Analysis of the immune response. Study of the activity of antiviral and virus neutralizing antibodies, quantitative determination of the levels of proinflammatory cytokines in the culture fluid from mMSCs and in the sera of animals, and the collection of splenocytes were carried out before and after infection with HSV-1: 14 days after the last immunization and 10 days after infection.

Detection of antibodies to HSV-1. Titers of HSV-1-specific antibodies were determined in mouse sera by ELISA, as described previously [23]. To detect HSV specific immunoglobulins of the IgG1 and IgG2a subclasses, 96-well plates were sensitized with iHSV at a volume of 100 μL/well at a concentration of 50 μg/mL overnight at room temperature. Then, serum samples were added at a dilution of 1 : 100 and incubated for 1 h at 37°C. The isotype of immunoglobulins bound to HSV-1 was determined using sheep anti-mouse antibodies conjugated with horseradish peroxidase (115-035-205 and 115-035-206, respectively; Jackson Immuno Research Lab, United States).

Virus neutralization assay. Neutralizing activity, the ability of antibodies to suppress HSV-1 replication, was determined on a Vero cell culture. Serum samples at various dilutions were incubated with HSV-1 (multiplicity of infection = 0.01 PFU/cell) for 2 h at 37°C. Then the reaction mixture was applied to a monolayer of Vero cells. The results were recorded after 48 hours, when the maximum CPE value was detected in the infected culture not treated with sera. The reciprocal of the maximum serum dilutions, at which a 50% suppression of the virus-specific CPE is observed, was taken as the activity of neutralizing antibodies.

Blast-transformation assay (BTA). The stimulation of proliferation of T-lymphocytes was judged by changes in cell morphology in BTA in vitro, by the formation of blasts. Splenocytes from mice in each group were pooled and plated in 24-well plates, at 10^5 cells per well. They was cultivated in growth medium RPMI-1640 containing 20% FBS (Invitrogene), 4.5 mg/mL glucose, 2 mM glutamine, 0.2 U/mL insulin, and 50 μg/mL gentamicin. The iHSV was added to the wells and used as a specific stimulator at a final concentration of 50 μg/mL, and growth medium without stimulants was added to assess spontaneous proliferation. Mitogenic concanavalin A (ConA, 5 μg/mL; Sigma, United States) was used as a positive control. The cells were incubated for 5 days at 37 °C in an atmosphere of 5% CO_2, after which the number of blasts in each well was counted (3 wells per variant) and the average values were calculated. The results were presented as the proliferation stimulation index (PSI), which was calculated using the formula $PSI = a/b$, where a is the number of antigen-specific blasts, and b is the number of non-specific (spontaneous) blasts.

Quantitative analysis of cytokines. Interleukins 2 and 6 (IL-2 and IL-6, respectively), interferon-γ (INF-γ), and tumor necrosis factor-α (TNF-α) were determined in cell culture fluids from primary MSCs and mMSCs in dynamics using the ELISA method. Secretion of IL-6, INF-γ and TNF-α in the mouse sera was assessed 14 days after the last immunization and 14 days after infection with HSV-1. The following test systems were used: Mouse IL-2 ELISA development kit (HRP), Mouse IL-6 ELISA development kit (HRP), Mouse IFNγ ELISA development kit (HRP), and Mouse TNF-α ELISA development kit (HRP) (Mabtech, Sweden). The sensitivity of the assays was 20 pg/mL for IL-6, 6 pg/mL for TNF-α and 4 pg/mL for IFNγ and IL-2. Cytokine concentrations were cal-

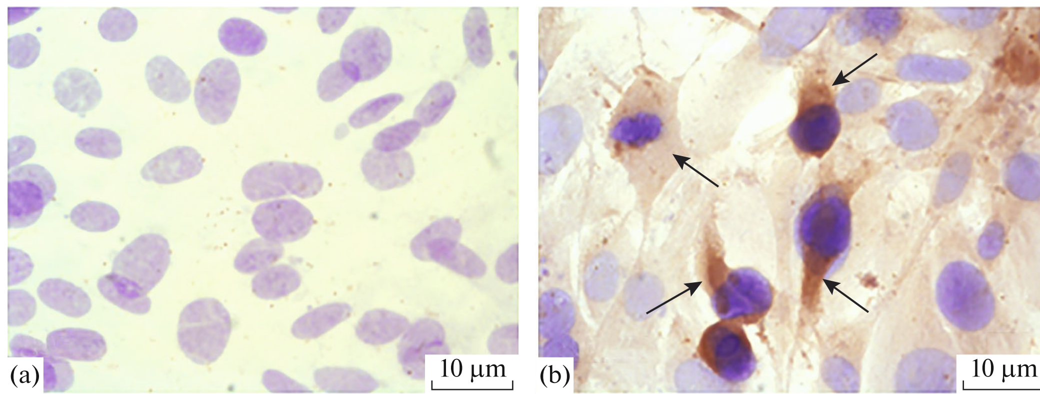

Fig. 1. Immunocytochemical detection of the gD protein of HSV in a genetically modified MSC transfected with the DNAgD plasmid. (a) Primary culture of MSC; (b) modified MSCs. The arrows indicate the accumulation of the gD protein in the cytoplasm of the transfected cells (brown staining). The nuclei are stained with hematoxylin (blue staining). Magnification 400×.

culated from the appropriate calibration curves according to the manufacturer's instructions.

Statistical processing of results. The results were statistically processed using the GraphPadPrism 5.1 and Statistica 6.0 software. For comparison of parametric quantitative data, we used the *t*-test (Student's *t*-test), for non-parametric data, the *U*-test (Mann–Whitney test) was used. Intergroup differences in relative indicators were analyzed using the χ^2 test (chi-square). Differences in indicators were considered statistically significant at $p < 0.05$. To assess the hypothesis about the relationship between the two variables, a correlation analysis was performed and the Spearman's rank correlation coefficient (r) was calculated. A value of $r > 0.7$ was considered as evidence of a strong relatioship.

RESULTS

Expression of the *Us6* gene of HSV was determined by immunocytochemical analysis using monoclonal antibodies to the gD protein 5 and 14 days after transfection of primary MSCs with the DNAgD plasmid and selection in the presence of geneticin G418. As a result of the counting of stained cells, it was shown that after 5 days, 75 ± 5% of the cells contained the viral protein (Figs. 1a, 1b), and after 14 days all cells produced the gD protein of HSV. The obtained mMSCs, stably producing the gD protein of HSV (MSCgD), were used to immunize animals.

The concentration of cytokines IL-2, IL-6, INF-γ, and TNF-α secreted by MSCs was determined in the cell culture liquids after transfection and in vitro selection in dynamics, starting from the 3rd day after transfection and ending by the 14th day, before the administration of mMSC to mice. The level of IL-2 decreased by day 3 after transfection, then increased on days 6–9, but by day 14, again, it strongly decreased: approximately 10-fold compared to the level of primary MSCs ($p < 0.05$) (Fig. 2a). In contrast, IL-6 secretion increased as early as by day 3 after transfection and remained at a high level, exceeding the level in primary MSCs by 9-fold by day 14 ($p < 0.05$) (Fig. 2b). The IFNγ concentration significantly increased on days 6 and 12 after transfection, then decreased by day 14 and was lower than the value for primary MSCs ($p = 0.02$) (Fig. 2c). TNF-α was not detected in the culture fluid from primary MSCs and mMSCs.

The humoral response to immunization with various immunogens was studied 14 days after the second immunization, as well as 10 days after infection with HSV-1. As a result of double immunization (Fig. 3a), the titer of HSV-1-specific antibodies in group 1 (MSC) was relatively low (1 : 200), their maximum production was detected in mice of group 4, which were immunized with iHSV (titer 1 : 14080). In group 2 (MSCgD), the content of antibodies was statistically significantly higher than in group 3 (DNAgD): titers 1 : 6400 versus 1: 1600 ($p < 0.001$). After immunization, antibodies of the IgG1 subclass prevailed in groups 1 (MSC) and 4 (iHSV) (Fig. 3b), whereas in groups 2 and 3 immunized with DNA or mMSCs, the ratio of antibodies of the IgG2a subclasses to IgG1 was significantly higher. After infection, in animals immunized with iHSV, antibody titers decreased, while in the remaining groups, especially in group 1, they increased significantly.

As for the neutralizing activity of antiviral antibodies produced by double-immunized animals, their titers in groups 1 and 2 (MSCs and mMSCs, respectively) were low, but statistically significantly higher than in other groups ($p < 0.05$). The same trend, but with much higher antibody titers, was found in the sera of infected animals.

The cellular response was studied in the lymphocyte blast-transformation assay in vitro. For this, splenocytes isolated from the spleen of mice were treated with iHSV to stimulate a specific response, ConA was used as a positive control, and the growth medium

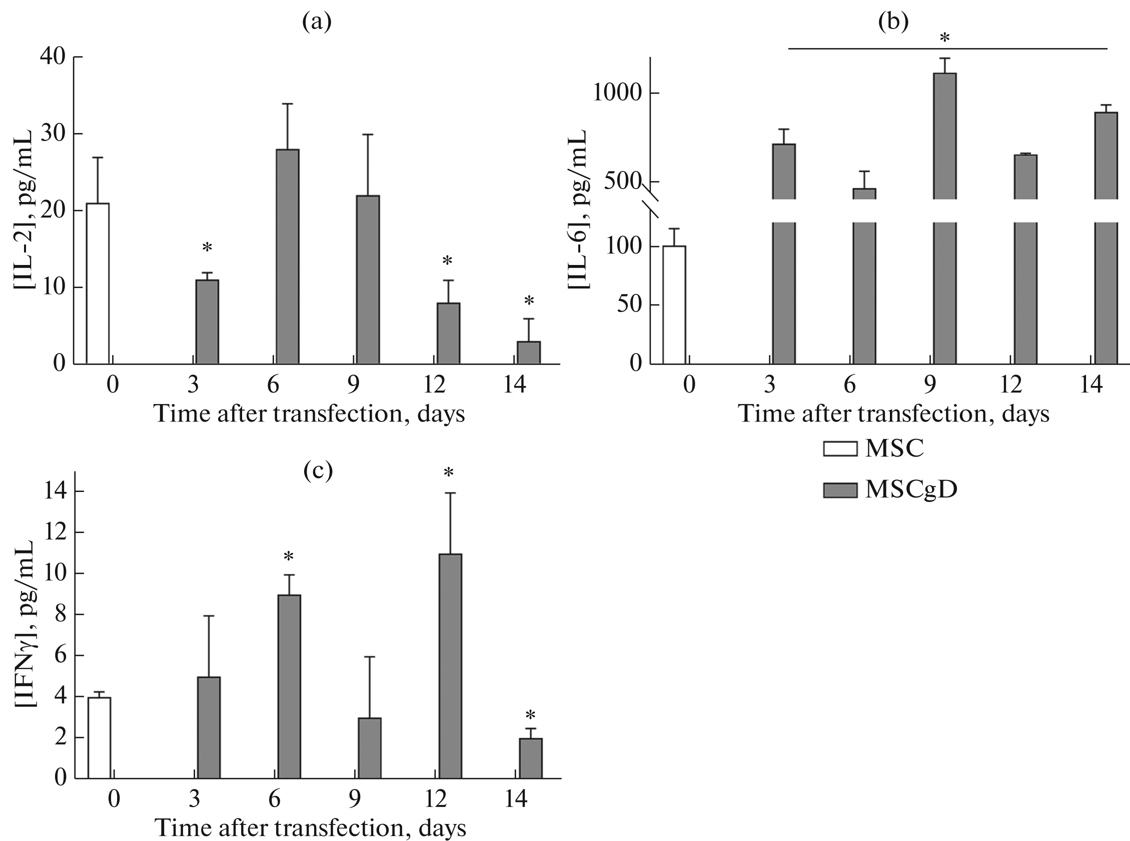

Fig. 2. Levels of the cytokines IL-2 (a), IL-6 (b), and IFNγ (c) secreted by primary and genetically modified MSCs in vitro. Results are presented as mean ± SD calculated from three replicates. $^*p < 0.05$ compared to MSC (*t*-test).

without stimulants which was utilized for cultivation of splenocytes was used to assess spontaneous (nonspecific) proliferation. It was found (Table 1) that the number of HSV-1-specific blasts was statistically significantly higher in all immunized groups compared to the control (group 5, $p < 0.05$), while the maximum number of blasts was stimulated by DNA immunization (group 3). Values of PSI (Fig. 4) for this group—6.6 before infection and 4.9 after infection—significantly differed from the PSI in other groups ($p < 0.05$). It should be noted that the level of spontaneous proliferation of splenocytes in animals immunized with primary MSCs (group 1), both before and after infection, was statistically significantly higher than in the other groups studied ($p < 0.05$) (Table 1).

Cytokines IL-6, IFNγ and TNF-α were detected in the sera of immunized mice in groups 1, 2, 3 and 5 before and after infection with HSV-1. As can be seen from the data in Table 2, after immunization, the level of IFNγ was significantly higher in group 1 (MSCs) compared to the other groups ($p < 0.05$), and in group 2 (mMSCs), it exceeded those for groups 3 (DNAgD) and 5 (control) ($p < 0.05$). After infection, the level of IFNγ in group 1 remained the highest, although in group 3 its concentration increased 23 times, and in control (group 5), 12 times. We did not study the levels of cytokines in animals of group 4, since, according to the literature, immunization with iHSV without adjuvants does not stimulate a significant increase in the level of IFNγ or the number of T cells producing this cytokine [24, 25].

The level of TNF-α before infection was statistically significantly lower in group 5 (control) compared to the experimental groups ($p < 0.05$); the concentrations of this cytokine in immunized animals did not differ significantly ($p > 0.05$). After infection, in mice of group 3, an almost twofold decrease in the level of TNF-α was observed, however, the differences were not statistically significant ($p > 0.05$). It should be noted that the cytokine concentration in this group was initially significantly lower than in other groups ($p < 0.05$).

Before infection, levels of IL-6 were low in groups 1 and 2 and undetectable in groups 3 and 5. In mice of group 3, IL-6 was practically absent in the blood serum both before and after infection.

The protective effect induced by various immunogens was studied within 14 days of infection with HSV-1 immunized mice in a model of systemic infection. In the control group, characteristic signs of herpes infection with lesions of the central nervous system——leth-

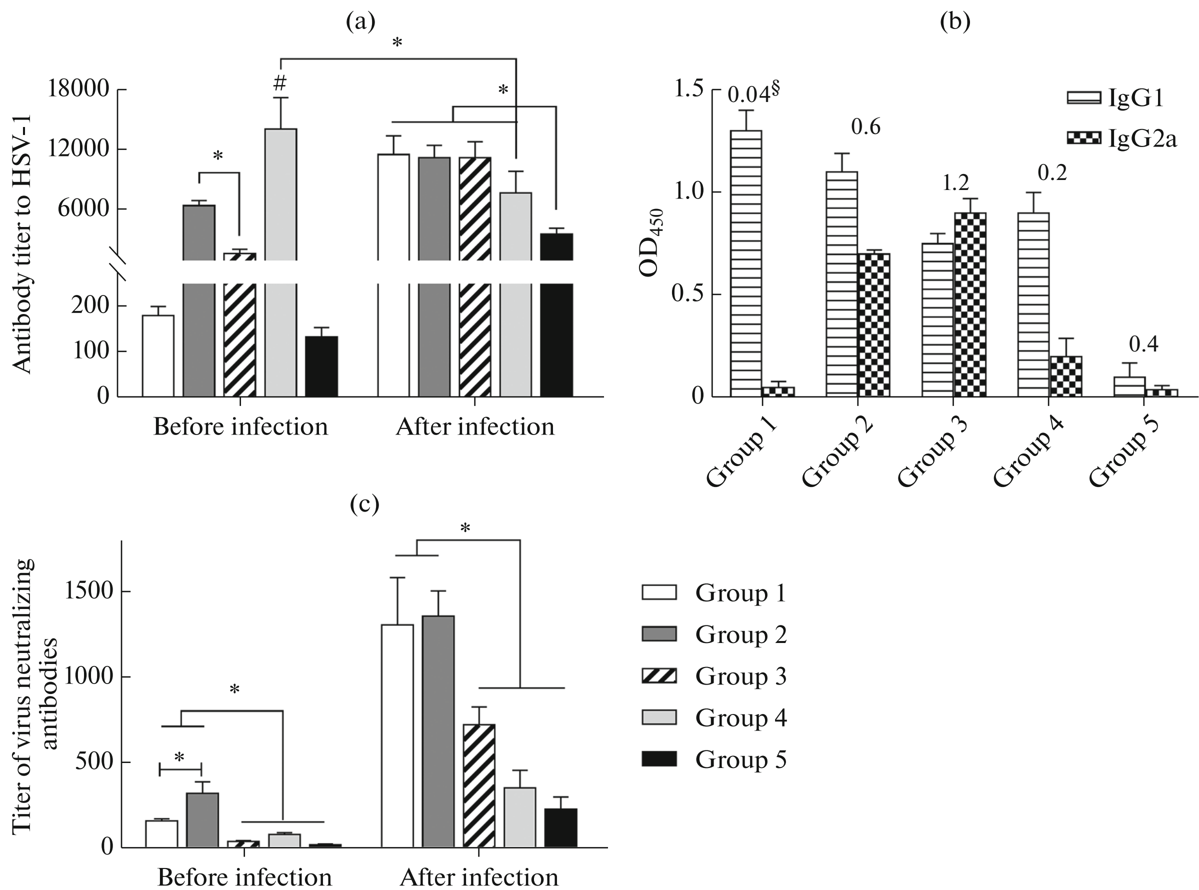

Fig. 3. Humoral immune response to immunization before and after infection of mice with lethal doses of HSV-1. (a) Titers of antiviral antibodies (ELISA); (b) relative levels of HSV-1-specific antibodies of IgG subclasses in immunized animals: group 1 (MSC), group 2 (MSCgD), group 3 (DNAgD), group 4 (iHSV), and group 5 (control). The results of ELISA are presented as absorbance values at 450 nm (OD_{450}). §Ratio IgG2a/IgG1. (c) Titers of neutralizing antibodies (virus neutralization assay); $*p < 0.05$, between groups (*t*-test); $\#p < 0.05$, compared to all groups (*t*-test).

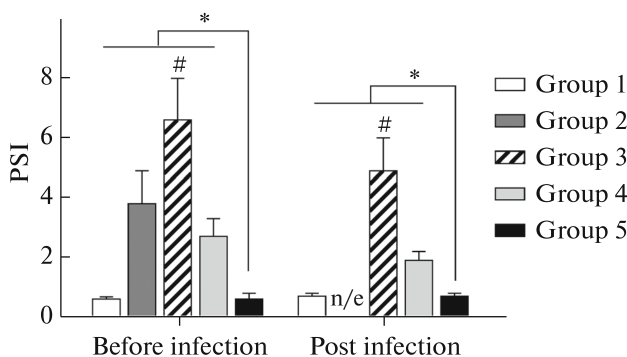

Fig. 4. Index of stimulation of splenocyte proliferation before and post infection of mice with lethal doses of HSV-1. The results are presented as mean ± SD, calculated for three replicates in immunized animals: group 1 (MSC), group 2 (MSCgD), group 3 (DNAgD), group 4 (iHPV), and group 5 (control); n/e—not examined. $*p < 0.05$, between groups (*t*-test); $\#p < 0.05$, compared to all groups (*t*-test).

argy, tousled coat, paresis of the hind limbs, and convulsions—were observed from the 5th day after infection, and by the 10th day all animals had died (Fig. 5). The survival rate of all immunized groups was statistically significantly higher compared to that of the control ($p < 0.05$). In group 1 (MSC), two mice developed neurological symptoms on day 6 after infection; however, by day 14, all animals had survived (100% survival). With mMSCs, 60% of mice survived (group 2), with DNA immunization (group 3), 65% (13/20), and with iHSV immunization (group 4), 50%. As a result of the statistical analysis, it was shown that the survival rate of animals in group 1 was significantly higher than in other groups ($p < 0.05$) and directly correlated with a high concentration of IFNγ (Table 2) and the titer of neutralizing antibodies (Fig. 3) ($r = 0.93/0.79$, respectively). Thus, it can be concluded that a double intravenous injection of primary MSCs protects 100% of animals from infection with 15 lethal doses of HSV-1.

Table 1. Proliferative activity of splenocytes in response to injected immunogens before and after infection of animals with lethal doses of HSV-1

Group	Number of blasts[a]					
	before infection			after infection		
	stimulants					
	ConA	iHSV	medium alone[b]	ConA	iHSV	medium alone
1 (MSC)	135 ± 6	32 ± 12	50 ± 17[c]	271 ± 76	92 ± 18	138 ± 28[b]
2 (MSCgD)	132 ± 6	54 ± 5	14 ± 2	n/e[d]		
3 (DNAgD)	122 ± 12	58 ± 4	9 ± 2	252 ± 41	130 ± 24	27 ± 8
4 (iHSV)	118 ± 8	31 ± 4	12 ± 3	238 ± 36	67 ± 20	35 ± 19
5 (control)	125 ± 19	9 ± 2[c]	8 ± 3	212 ± 52	22 ± 7[c]	25 ± 9

[a] Number of blasts detected in BTA in vitro. Results are presented as mean ± SD (data from triple replicates); [b] growth medium without stimulants; [c] $p < 0.05$ compared with other groups in each column (t-test); [d] not examined.

Table 2. Levels of proinflammatory cytokines in the sera of immunized mice before and after infection with HSV-1

Group	Cytokine concentration, median [minimum; maximum], pg/mL					
	before infection			after infection with HSV-1		
	IL-6	IFNγ	TNF-α	IL-6	IFNγ	TNF-α
1 (MSC)	11 [4; 68]	209[a] [98; 396]	24 [4; 93]	13 [4; 55]	236[a] [103; 468]	38 [12; 120]
2 (MSCgD)	20 [4; 83]	63[b] [35; 132]	11 [4, 20]	n/e[c]	n/e	n/e
4 (iHSV)	n/e[c]					
3 (DNAgD)	<4[a]	4 [1, 18]	19 [10; 55]	< 4[a]	93 [36; 129]	10[a] [8, 25]
5 (control)	<4[a]	2 [1, 6]	5[a] [1, 11]	14 [4; 67]	48 [1; 98]	28 [1; 102]

[a] $p < 0.05$ compared to all groups in each column (U-test;); [b] $p < 0.05$ compared with groups 3 and 5 (U-test); [c] not examined

DISCUSSION

The widespread prevalence of herpesvirus infections, despite the use of virus-specific therapy, means that vaccines must be used to control these diseases. However, 60 years of research in this area has not yet been crowned with success. From this it is inferred that it is necessary to introduce new strategies to solve the problems associated with diseases of herpesvirus etiology. We have investigated the possibility of using MSCs as a candidate prophylactic vaccine against HSV infection. MSCs are a heterogeneous population of stromal stem cells that can be obtained in large quantities from various sources: bone marrow, adipose tissue, umbilical tubule, dental pulp, menstrual blood, and even skin. The multipotency, immunoregulatory and regenerative properties of MSCs, as well as the lack of immunogenicity, led to the initiation of studies on the use of MSCs in the treatment of diseases of various etiologies. The safety of MSC therapy with intravenous administration has been proven [27]. International organizations such as the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have approved the use of certain MSC-

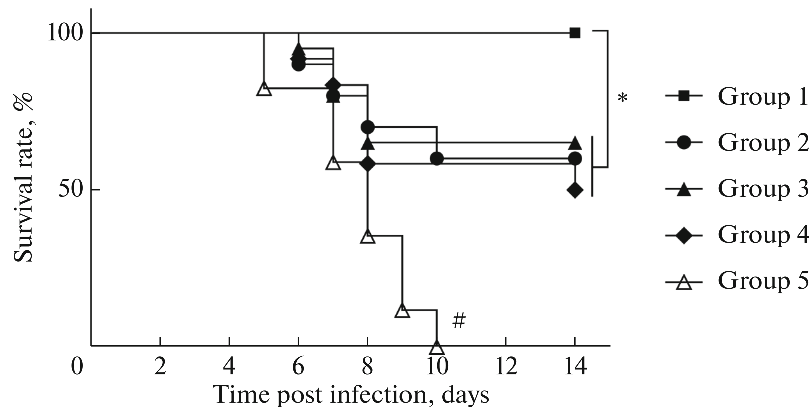

Fig. 5. Protective response during experimental systemic HSV-1 infection in mice immunized with MSCs (group 1), mMSCs (group 2), DNAgD (group 3), or iHSV (group 4), and in the control group (group 5). * $p < 0.05$, # $p < 0.05$, survival in control compared with immunized groups (Kaplan–Meier estimator).

based products for the treatment of Crohn's disease and post-transplant Graft-versus-host disease (GvHD) [28, 29].

In works aimed at creating vaccines against herpes using models of HSV infection, it was concluded that there is no direct correlation between immunity and protection [30], a high immune response cannot be a guarantee of a protective effect. Considering these findings, we immunized mice with the selected immunogens and evaluated the prophylactic effect in vivo, after infecting animals with lethal doses of HSV-1. For reference, we used: (1) primary (native) MSCs; (2) MSCs transfected with a plasmid containing the *Us6* gene encoding the gD protein of HSV; (3) recombinant DNA containing the *Us6* gene of HSV, and (4) whole-virion inactivated HSV-1. It should be noted that the last two immunogens are included in the candidate vaccines currently being tested: a DNA vaccine that stimulates a cellular response [31]; and an inactivated viral vaccine that prevents HSV-2 infection and disease in guinea pigs [32]. As a result of our experiments, we have shown that complete protection from lethal HSV-1 infection is provided by two-fold administration of primary MSCs, all other immunogens were less effective—they protected 50–65% of animals from death (Fig. 5).

It was of interest to compare the parameters of the immune response of animals. As a result of immunization (before infection), iHSV (group 4) stimulated the antibody response to HSV-1 and a weak cellular response, while DNAgD (group 3) stimulated a weak humoral and strong T cell response. These data are consistent with the results from other authors obtained earlier in different experimental models when comparing the prophylactic effect of subunit/viral and DNA vaccines (see review [33]). Primary MSCs after double injection induced the production of HSV-1-specific antibodies, albeit in low titers, and stimulated innate immunity, judging by the high level of IFNγ. The mMSCs expressing the *Us6* gene of HSV induced both an antibody response to a viral antigen and a cellular immune response. At the same time, when immunized with mMSCs, the antibody titer was lower than during immunization with iHSV, and the level of lymphocyte proliferation (PSI) was lower than during DNA immunization (DNAgD). After infection, the antibody response increased in all animals, except for group 4, immunized with iHSV, where the level of antibodies decreased. It is important that in animals immunized with MSCs and mMSCs, the titers of neutralizing antibodies were significantly higher than in the other groups. The greatest differences between the groups were found in the concentration of IFNγ, which was highest in the blood of animals immunized with primary MSCs. It is noteworthy that after immunization with primary MSCs, there was no statistically significant proliferative response of T cells to HSV-1 with the increased level of spontaneous (nonspecific) response, both before and after infection with HSV-1. At the same time, in both cases, a high level of IFNγ production was recorded (Tables 1 and 2). The nonspecific response of T cells after a two-fold injection with MSCs is possibly associated with the functional features of these cells, the generation and proliferation of which is induced by environmental cytokines, regardless of the foreign antigen [34]. These cells have a 'memory-like' phenotype like memory T cells. It has been shown that type I IFNs (IFN-α/β), produced under homeostatic conditions or during infection, promote the proliferation and functioning of 'memory-like' $CD8^+$ T cells [35]. T cells with a broader specificity as components of a heterologous immune response—with nonspecific, but beneficial effects—can significantly improve the immune response, which should be taken into account when creating vaccines [34].

It should be noted that antibodies detected after immunization with primary MSCs (group 1) were

mainly of the IgG1 subtype (a marker of the Th2 response), and the ratio of antiviral antibodies of subtypes IgG2a (marker of Th1 response)/IgG1 in this group was minimal. According to the prevailing views, this means that the immune response is biased towards Th2 cells and is aimed at the synthesis of anti-inflammatory cytokines (IL-4, IL-10, etc.). As a result of our experiments, we revealed high concentrations of proinflammatory cytokines, especially IFN-γ, produced mainly by cells of the Th1 phenotype. It can be assumed that this 'contradiction' is explained by the fact that MSCs secrete a large set of both pro- and anti-inflammatory cytokines [36], which are capable of activating a wide range of immune cells. In addition, immune cells induced by MSCs can modify the type of response from humoral Th2 to cellular Th1 and stimulate the production of IFN-γ [37]. Apparently, due to the listed properties of MSCs, after infection of animals immunized with them, both the T cell and B cell responses to HSV-1 are activated, and also protection against lethal infection is formed. The more powerful protective effect of primary MSCs than mMSCs may be due to the fact that, as a result of transfection and subsequent selection of cells expressing the introduced *Us6* gene of HSV, the secretion of proinflammatory cytokines in mMSCs changes in vitro, i.e. the production of IFN-γ and IL-2 drops significantly and the level of IL-6 increases substantially (Fig. 2). Introduction of the selected mMSCs led to a change in the concentration of cytokines in the blood of immunized mice as well: the concentration of IFN-γ decreased approximately 2-fold and the concentration of IL-6 increased 2-fold. An increase in the level of IL-6 is recorded during various inflammatory diseases and is considered a marker of the development of severe forms of diseases, including herpesvirus etiology [38] and COVID-19 [39]. Apparently, such changes in the cytokine pattern contribute to a decrease in the protective activity of the mMSC-immunogen compared to MSCs in the experimental model of HSV-1 infection, since when vaccinated with primary MSCs, which induce 100% survival of mice, the concentration of IL-6 did not change, but IFN-γ increased.

Most of the published works describe the immunosuppressive properties of MSCs (see review [37]), due to which they suppress excessive inflammatory reactions, cytokine storm, and transplant rejection. Other studies have shown that MSCs have immunoregulatory rather than immunosuppressive properties and can not only suppress, but also stimulate immune responses [40]. In the body, MSCs are present in almost all tissues and express many types of receptors, therefore differences in the direction of their regulatory functions can be explained by differences in their microenvironment [41]. Under conditions of inflammation, at high levels of the pro-inflammatory cytokines IFN-γ and TNF-α, MSCs are activated and acquire an immunosuppressive phenotype—MSC2. In the absence of signs of inflammation in the microenvironment (low levels of TNF-α and IFN-γ), MSCs acquire a pro-inflammatory, MSC1, phenotype and enhance the T cell response. MSC activation was studied in dynamics, after the addition of poly(I:C) as an analog of double-stranded viral RNA [42]. The authors found that the properties of MSCs during activation can change from pro-inflammatory to anti-inflammatory, and they defined this as the dependence of polarization on the time of action of MSCs, the time-dependent MSC polarization. In our experiments, MSCs were injected into healthy animals, and this was one microenvironment, and after infection with the virus, the microenvironment of MSCs changed, which led to stimulation of the B cell response and, especially importantly, to a significant increase in the level of virus neutralizing antibodies. These results are in good agreement with the data obtained by Glenn & Whartenby [43] when studying the effect of MSCs on B cells in vitro. Under conditions mimicking a viral infection, the presence of MSCs increased the survival of B cells, their differentiation, and the levels of antibodies they produced.

Infection of mice with HSV-1 after immunization with MSCs, apparently, triggers many mechanisms, including both antigen-dependent and antigen-independent ones. The molecular and cellular mechanisms that provide antiviral action under these conditions are being actively studied [44–46] and will hopefully be revealed in further studies.

According to recent data, the successful therapeutic effect of MSCs may be long-term [47], which suggests the induction of memory T cells, which are necessary for the protective effect of the vaccine [48]. It can be assumed that after two-fold immunization with MSCs, memory cells were formed in mice, the functioning of which contributed to the complete protection of the animals from the subsequent lethal viral infection.

Thus, due to the unique ability of MSCs to induce innate, adaptive and protective immunity, as well as their availability and safety, these cells can be considered in the future as the main components of new generation vaccines (based on cellular technologies) against herpes and other infections.

FUNDING

This work was financially supported by a grant from the Russian Foundation for Basic Research (No. 19-04-01218).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. In carrying out this work, all ethical standards were observed. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

This article does not contain any studies involving human participants performed by any of the authors.

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Translated by A. Ostyak