THE STUDY OF LEUKOCYTE PHAGOCYTIC ACTIVITY IN THE PRESENCE OF HERPETIC INFECTION AND STROKE

BADANIE AKTYWNOŚCI FAGOCYTARNEJ LEUKOCYTÓW PRZY WSPÓŁISTNIENIU ZAKAŻENIA OPRYSZCZKĄ I UDARU

Natalia V. Motorna[1], Svetlana L. Rybalko[2], Daria B. Starosyla[2], Mykhailo M. Guzyk[3], Iryna G. Strokina[1], Rostyslav F. Kaminsky[1], Alina V. Korsak[1], Sergey I. Savosko[1], Liudmyla M. Sokurenko[1], Yurii B. Chaikovsky[1]

[1] BOGOMOLETS NATIONAL MEDICAL UNIVERSITY, KYIV, UKRAINE

[2] GROMASHEVSKY L. V. INSTITUTE OF EPIDEMIOLOGY AND INFECTION DISEASES, NAMS OF UKRAINE, KYIV, UKRAINE

[3] PALLADIN INSTITUTE OF BIOCHEMISTRY OF THE NATIONAL ACADEMY OF SCIENCES OF UKRAINE (NASU), KYIV, UKRAINE

ABSTRACT

Inrtoduction: Post-stroke complications are one of the urgent and insufficiently resolved problems. According to different literature data 23% to 65% of patients suffer from the post-stroke development of an infectious process. Herpes simplex virus type 1 and 2 can also be etiological factors of stroke development, however their reactivation is seldom mentioned in clinical observations. The development of immune suppression is considered to be the cause of these complications.

The aim: The current study aims at determining post-stroke changes in leukocyte component of the immunity and in the presence of concomitant herpetic infection as well as at finding changes in phagocytosis parameters during antiviral treatment.

Materials and methods: The experiments were carried out on mice of the Balb/c line. The animals were infected with the herpes simplex virus type I, and 30 days later hemorrhagic stroke was simulated by administering 0.1 ml of autoblood into the right hemisphere. Following the acute stroke some animals were given acyclovir, proteflazid or altabor. From the animals' blood leukocytes were obtained and phagocytic activity and production of reactive oxygen species of granulocytes and agranulocytes in relation to fluorescent *E.coli* bacteria were studied by flow cytometry.

Results: The experiment revealed significant changes in the redistribution between two major types of leukocytes in mice with stroke (an increased number of agranulocytes by 19.9%) and decreased phagocytosis activity, in the animals infected with herpes simplex virus type I in particular. Ischemic brain damage had an immunosuppressive effect on blood leukocytes. For comparison a significant increase in phagocyte count in leukocytes was found in the case of viral infection. The use of drugs with antiviral effects did not affect the activity of granulocytes / agranulocytes.

Conclusion: Stroke can be the cause of latent herpes virus infection reactivation and has essential negative effect on immune characteristics of leukocytes that remain unchanged with the use of antiviral agents.

KEY WORDS: stroke, herpes simplex virus type I, leucocytes, acyclovir, proteflazid, altabor.

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INTRODUCTION

Stroke is one of the main causes of disability and death around the world [1]. With the increased incidence of stroke the growing number of its concurrent complications is being registered. One of the most prevalent complications of the stroke is infection. Clinical and experimental studies show that stroke impairs interaction between the central nervous system and the immune system, leading to the development of bacterial and viral infections [2,3,4,5].

According to different literature data 23% to 65% of patients suffer from the post-stroke development of an infectious process, with pneumonia and urinary tract infections being the most common [6]. The most frequently found bacterial agents include pathogens from *Streptococcus*, *Klebsiella*, and *Staphylococcus* genii [7]. Although the authors believe that a large proportion of infectious diseases has viral etiology, they are not always diagnosed and managed appropriately [8]. Herpetic infections belong to viral agents that are often mentioned in clinical research. Herpes simplex virus type 1 and 2 can also be etiological factors of stroke development, however their reactivation is seldom mentioned in clinical observations [9,10]. The development of immune suppression is considered to be the cause of these complications.

More detailed research of pathophysiological processes occurring after the stroke is needed for determining potential therapeutic targets to improve the treatment outcomes and prevent complications of the disease. Known, for different diseases the first indicator is the study of blood [11].

THE AIM

This study aimed at determining post-stroke changes in leukocyte component of the immunity and in the presence of concomitant herpetic infection as well as at finding changes in phagocytosis parameters during antiviral treatment.

MATERIALS AND METHODS

The experiments were conducted on BALB line mice weighing 18-20 g. The animals were kept under controlled conditions: temperature (22.0±2.0°C), humidity (55.0±5.0%) and photoperiod with free access to conventional granulated combined feed and drinking water.

The experimental animals were infected by epidural injection of viral material (freeze-dried HSV type I VC) in the retroorbital area. The development of infectious state symptoms in the control group was registered on day 5-6, followed by reduction of infection manifestations severity and ultimate recovery of the animals. Since that point of time HSV-1 has been transferring into its latent form. This model is convenient for evaluating symptoms manifestation, is characterized by 100% reproducibility and does not require any additional methods of control.

On day 30 after the resolution of viral infection manifestations (weakness, decreased agility, decreased need in food and water) hemorrhagic stroke was simulated in survivors. Simulation of a limited hemorrhage (intracerebral hematoma) in the animals' brain was achieved by administration of 0,1 ml of autoblood to the right hemisphere (L=1,5, H=3,0, AP=1,0) [12].

In all animals the haematoma was located in the right internal capsule (capsula interna dextra) of the brain. It allowed to reproduce the standardized model of intracerebral haematoma that does not require additional verification of the type, volume and localization of the lesion and the corresponding sequelae of the pathological condition were similar in all groups of animals and consistent with the standardization and methodology of the experimental study.

The comparison groups were represented by intact animals, animals with stroke and animals with HSV-I. Antiviral drugs proteflazid (37,2 mg/ml), acyclovir (50 mg/ml) and altabor (5 mg/ml) have been administered intraperitoneally to a part of animals for 10 days after infection and stroke simulation.

The experimental groups were the following:

Group 1 – control intact animals (n=6)

Group 2 – animals with stroke (n=7)

Group 3 – animals with HSV-I (n=7)

Group 4 – animals with HSV-I and stroke (n=7)

Group 5 – animals with HSV-I and stroke+proteflazid (n=6)

Group 6 – animals with HSV-I and stroke+acyclovir (n=6)

Group 7 – animals with HSV-I and stroke+altabor (n=6)

The presence of HSV-I in the infected animals (brain, blood, liver) was proved by dot-ELISA assay and *in vitro* method (in Vero cell culture) (n=3, from all groups).

The animal's blood was drawn in the morning after fasting (for 12 hrs) under light ether anesthesia. To prevent blood clotting heparin was used (50 MO). The leucocytes were obtained from peripheral blood of experimental animals by hemolysis of erythrocytes on the day of experiment. For this purpose 5,0 ml of distilled water were added to 100 μl of peripheral blood followed by addition of 5,0 ml of 1,8% of NaCl solution 20 seconds later. The cells have been spinned down at 400g (R18, 1410 rpm) at 12°C for 5 min and the procedure was repeated one more time. The supernatant was poured off and the volume added up to 10 ml by normal saline. Then the cells have been spinned down for 400 g (R18, 1410 rpm) at 12°C for 5 min. Supernatant was poured off and the cells were resuspended in 500 μl of phosphate-buffered saline (PBS) (pH 7,2).

The production of reactive oxygen species (ROS) by leukocytes was measured using 2',7'-dichlorofluorescin diacetate (2',7'-Dichlorofluorescin diacetate MW 485.27, 35845, Sigma-Aldrich) in final concentration of 25 μM according to the methodology [13]. After incubation (+ 37°C) for 45 min in the place protected from sun rays the cells were washed in 10 ml PBS buffer and spinned down by centrifugation at 400 g (R18, 1410 rpm) at 12°C for 5 min. The pellet was resuspended in 500 μl of PBS. The intensity of the studied samples emission was registered by channel logFL1 (515-535 nm) in flowcytometer COULTER EPICS XL (Beckman Coulter, USA), equipped with argon laser (λ of excitation = 488 nm).

The redistribution among different populations of leukocytes was assessed using two parameters of COULTER EPICS XL flow cytometer (Beckman Coulter, USA), by the value of forward (FS, size of the cells) and side light scattering (SS, granularity of cells).

The percentage of phagocytozing cells and engulfing of live fluorescent bacteria by macrophages in samples was evaluated using live fluorescent bacteria *E. coli* following methodology similar to the described one [14]. The cells have been incubated for 90 min at 37 °C with live fluorescent bacteria *E. coli* in concentration of 6×10^6 bacteria per ml. Flowcytometry on COULTER EPICS XL (Beckman Coulter, USA) by channel log FL1 (515-535 nm) was conducted after washing.

The principle of phagocytic activity method is based on engulfing of *E. coli*, expressing green fluorescent protein (Green Fluorescent Protein, GFP) by neutrophils, monocytes and macrophages (by phagocytosis). This protein has mol. weight of 26,9 kDa and fluoresces with green light when excited by blue light (laser, 488 nm). Accordingly, the more bacteria have been engulfed by the cell the higher the fluorescence intensity of GFP.

More than 10000 occurrences from each sample were analyzed. The results were processed using program FCS Express V3.

Statistical processing of the obtained samples was conducted using program Statistica 6.0. The samples were compared using the Student's test. Results were presented as a mean (M) and standard deviation of the mean (± m). The difference was considered to be statistically significant at $p < 0,05$.

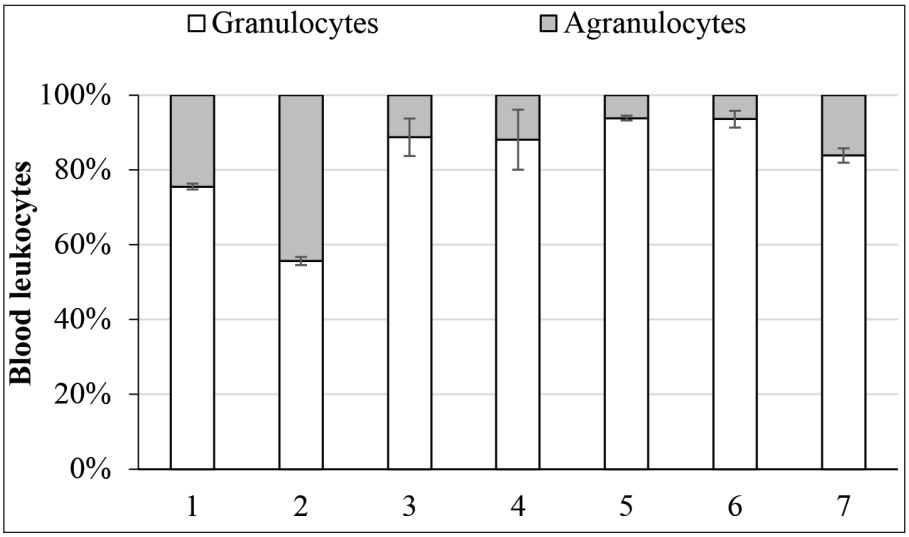

Fig. 1. Redistribution of blood leukocytes in groups of experimental animals (n=5). * - parameters differ from the group 1 (P<0,05) # - parameters differ from the group 2 (P<0,05)

RESULTS AND DISCUSSION

Significant difference in distribution between two main types of leukocytes was found in experimental conditions (Fig. 1). Animals with stroke (group 2) were characterized by a shift in leukocyte ratio to the agranular leukocyte side. Average ratio between leukocytes has shifted by 19,9% to the agranulocyte side (p<0,05).

The number of granulocytes increased in groups of animals with HSV-I (group 3) by 13,2% (p=0,05) on the average and by 33,1% (p<0,05) as compared to group 2.

In groups with HSV and stroke (groups 4-7) the granulocytes/agranulocytes ratio did not differ from data of group 3 and was significantly higher compared to group 2.

The decreased fluorescence intensity of 2',7'-dichlorofluorescein diacetate in granulocytes of animals with stroke (group 2) was found as compared to control, moreover this value did not differ from control or even had tendency to increase in groups infected with HSV-I as it was shown in groups 4-7 (Fig. 2). There was no sufficient difference between comparison groups 4-7. A slight decrease of granulocytes fluorescence intensity (8,6%, p<0,05) was shown for group 5 as compared to group 4.

Agranulocytes were characterised by increased fluorescence intensity in all groups with HSV- I (groups 3-7) as compared to control. Fluorescence intensity of 2',7'-dichlorofluorescein diacetate is considered to be directly proportional to the content of such ROS as hydroxyl radical OH^- and peroxynitrite ($ONOO^-$) in cells. Thus, the mentioned increase of the studied value is a manifestation of intensification of ROS production in leukocytes in the presence of HSV-I.

The fluorescence rate in leucocytes from groups 4-7 did not statistically differ from that in group 3. It indicates the effect of HSV- I on functional state of leucocytes and their production of ROS.

While evaluating phagocytic index (ratio of phagocytozing cells to the total amount of cells in the studied leukocyte samples) we have observed its decrease in groups of animals with the simulated stroke. In HSV-I group the phagocytic index did not differ from the control level. These data show that ischemic damage of brain causes immune-suppressive effect on leukocytes.

Analysis of phagocytic number change, i.e. the number of phagocytosed units of *E.coli* by a single leukocyte also confirms the phenomenon of immune suppression in the stroke group (the decreased fluorescence intensity by 39,1%, p<0,05). Moreover, a notable increase of phagocytic index was shown for the group of animals with HSV-I (group 3). These parameters in group 4 did not significantly differ statistically from groups 1 and 3, this is the case with groups 5 and 7 that were administered proteflazid and altabor where that index had also increased.

Thus, the stroke causes immune suppression which is manifested by the decrease of granular leukocytes count and their phagocytic activity. Immune activity of leukocytes in the presence of HSV-I includes the increase of granular leukocyte count and production of ROS by agranulocytes. Stroke with underlying HSV-I has shifted leukocytes ratio to granulocyte side, which were characterized by the dramatic drop of phagocytosis while the decreased number of agranular leukocytes did not differ from the control by the phagocytic number. In the groups, which have received the medicine the leukocyte parameters did not differ from the comparison group and the phagocytic number in groups 6 and 7 was even lower.

Our experimental study has demonstrated the connection between stroke, herpes virus infection and immune disorders. Herpes virus infection is the most common among viral infections and is often latent. Clinical research has shown the connection between blood vessels damage and the development of stroke in the presence of latent or acute form of herpes virus infection [15,16].

In this study an attempt to find a causal relation between stroke and infection reactivation was made. For that purpose stroke was simulated in the animals infected with strain of the virus from the museum (which was deliberately adapted to the research on laboratory mice) followed by the assessment of the functional parameters of leukocytes to identify manifestations of immune suppression. As shown by the study results, the stroke is accompanied

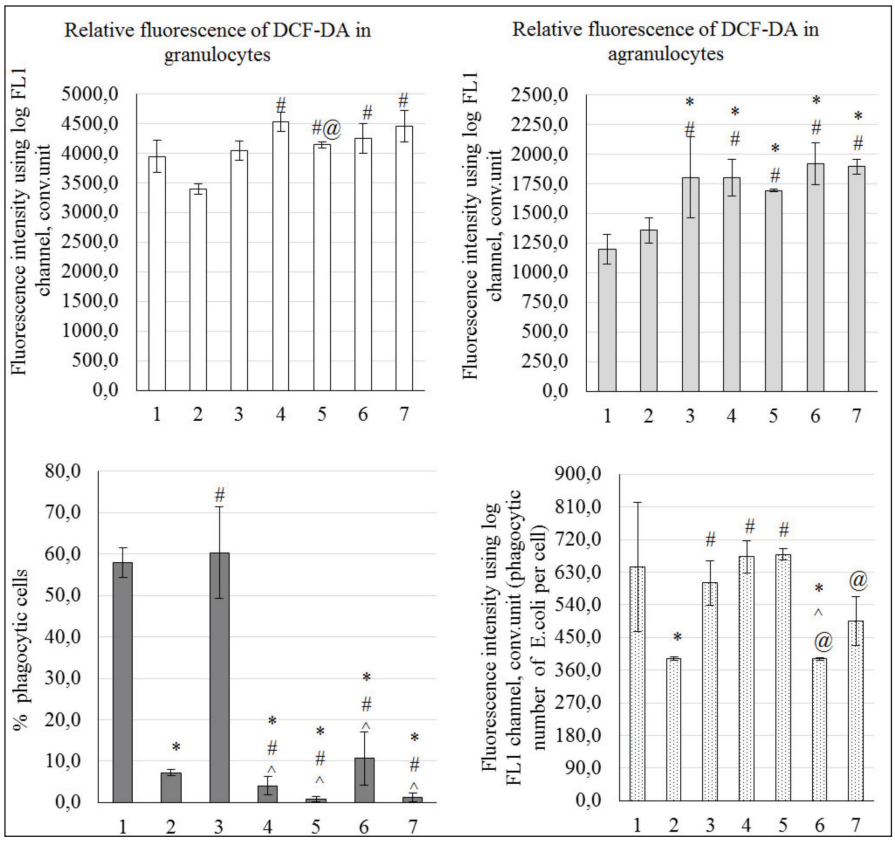

Fig. 2. Functional features of granulocytes and agranulocytes in mice blood
* - parameters differ from the group 1 (P<0,05)
# - parameters differ from the group 2 (P<0,05)
^ - parameters differ from group 3 (P<0,05)
@ - parameters differ from the group 4 (P<0,05)

by a significant reduction of the total granulocyte count, their ROS-producing function and phagocytic activity. Similar changes were found in infected animals with stroke. These findings give evidence of the development of post-stroke immune suppression. Moreover there was no significant difference found between groups of animals, which were given antiviral agents and comparison group which had not received any agents. These findings provide additional evidence of the significant negative effect of the stroke on the immune system whereas the impact on the infectious process does not contribute to the recovery of the functional activity of leukocytes.

Unfortunately, the mechanism of post-stroke immune suppression remains unclear. Studies of these disorders in the setting of stroke are focused on two main areas: local immune responses and systemic infections [17]. Different immune cells play different roles in stroke development. Macrophages and monocytes are known to cross the blood-brain barrier and release inflammatory mediators thus increasing infiltration by other leukocytes [18]. Monocytes and macrophages are the main phagocytes and antigen-presenting cells that initiate subsequent immune responses by interacting with T-cells [19]. The authors believe that in case of brain ischemia monocytes utilize the damaged nervous cells thus facilitating regenerative processes. However, simultaneous or concurrent infiltration by neutrophils has negative effect on the extent of brain damage and progression of inflammation [20]. Neutrophils quickly respond to the infectious agent realizing their immune function by generation of superoxide [21]. Impairment of this function of neutrophils was noted in patients with ischemic and hemorrhagic stroke, which explains the decreased resistance to infections [22].

The pro- and anti-inflammatory mechanisms balance can be directed at limiting the degree of inflammation and promotes reconstitution of immune homeostasis. However there are no doubts that inflammatory processes at the site of ischemia lead to adverse outcomes, development of neurological disorders especially in the presence of co-infections.

Results of clinical researches have demonstrated the low level of lymphocytes, functional deactivation of monocytes, T-helpers and NK-cells during first 6 days after the stroke [23,24], with resistant lymphopenia found in patients with inflammation [25]. Furthermore the decrease of pro-inflammatory cytokines level can also be a factor of the emergence of infection [26]. Another proof of systemic immune disorders is the shrinking of immune organs (spleen, thymus) that was demonstrated in experimental research [27]. These disorders are considered to be the result of hypothalamic-pituitary-adrenal system response, release of adrenocorticotropin and ultimate acute increase of adrenocorticoids in blood [28,29]. Similarly the stroke-induced activation of the sympathetic nervous system leads to secretion of catecholamines by adrenal medulla and nerve endings [30]. Immune cells respond to glucocorticoids and catecholamines and extra stimulation causes apoptosis of lymphocytes, which explains the post-stroke immunodeficiency and other pathological conditions [31,32]. Lymphopenia becomes one of the causes of immune suppression.

CONCLUSION

Thus, stroke can be the cause of latent herpes virus infection reactivation, has essential negative effect on immune characteristics of leukocytes that remain unchanged with the use of antiviral agents.

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ADDRESS FOR CORRESPONDENCE
Liudmyla Sokurenko
Department Histology and Embryology
of National O.O. Bogomolets Medical University
tel. +380675010815.
e-mail: l-sokurenko@i.ua

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