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MURINE SPLEEN LYMPHOCYTES APOPTOSIS IN THE EXPERIMENTAL MODEL OF STAPHYLOCOCCAL INTRAVAGINAL INFECTION

The results of research on changes in the level of apoptosis among the spleen cells during the development of intravaginal staphylococcal infection in experimental models of different genetic mice lines are given in the paper. Intravaginal injection of Staphylococcus aureus resulted in an increased number of cells with morphological characteristics of apoptosis in underbred and line BALB/c mice, while it decreased in the ICR line mice. The level of the apoptotic cells in the underbred and line BALB/c mice was accompanied by some level of microorganisms that were seeded out of kidneys – the higher was the number of microorganisms, the more apoptotic cells were indicated.

K e y w o r d: apoptosis, spleen, Staphylococcus aureus, mice lines.

Infectious-inflammatory diseases caused by pathogenic and opportunistic strains of staphylococci, are growing over the past two decades. It becomes increasingly important to study the role of staphylococcal infections in the general pathology of the reproductive system and pregnancy [17],

which are characterized by numerous violations of the factors of congenital and acquired immunity [9]. It is known that an important role in the development of immunodeficiency states which accompany various pathological processes, including the bacterial infections, is given to the violation of apoptosis regulation in the immune system cells [5].

It was determined that apoptotic program in the lymphocytes can be triggered by high doses of specific antigen as well as by the microbial superantigen, accompanied in the first case by the development of immune tolerance, and in the second one – by formation of immunosuppressive condition [13].

Viral and bacterial antigens, toxins, cytokines, including tumor necrosis factor, neurotransmitters, hormones, free radicals, oxidative stress, loss of contact between the cells, temperature, and various types of radiation, antibiotics, ethanol, monoclonal antibodies and other specific ligands can be the external inducers of apoptosis [12].

It is logicaly to consider the apoptosis as a part of immunogenetic control of the body homeostasis provided by the reparatory enzymes system, immune system and in extreme situations – enabling genes programmed death if the reparation by other means is impossible [14, 18]. However, reducing of the apoptosis intensity also leads to negative consequences, such as autoimmune diseases and malignant tumors [7]. It is known that *Staphylococcus* antigens may also induce apoptosis, but the exact mechanism has not yet been clarified [6].

In connection with the foregoing, the research aim was to determine the impact of intravaginal staphylococcus infection on the immune system cells apoptosis in experimental models of mice of different genetic lines by studying the number of cells in state of apoptosis among splenocytes.

Materials and Methods. The objects of the study were white laboratory female mice of ICR, BALB/c lines and underbred aged ones from three to seven months and 15-21 g weight, kept on standard vivarium diet. The mice were handled in accordance with generally accepted international rules of the work on experimental animals. The BALB/c line mice came from the white commercial mice Beggz (1906) and are used in the oncology and neuroscience research. The ICR line came from a large colony of Swiss white mice and is used mainly in pharmacological studies of drugs [20].

Staphylococcal urogenital tract infection was depicted by intravaginal injection of exclusive mice extracted cell suspension of *S. aureus* in a dose of 1×10^{10} cells/ml. The strain of *S. aureus* was isolated from urogenital tract of intact underbred white mice at the Department of Microbiology and Virology of O. Gonchar Dnipropetrovsk National University [2].

Splenocytes were obtained by the mechanical destruction of spleen in the growth media 199 or RPMI-1640 with following centrifugation for 10 minutes at 1500 rpm. Supernatant was completely removed and the cells were resuspended in 1 ml of growth media [19].

Lymphocytes were isolated from spleen cell suspensions by fractionation in density gradient of fikoll-verografin ($\rho = 1.12$ and $\rho = 1.077$ g/cm^3). The cell suspension was laminated carefully on the top of two-step gradient of fikoll-verografin. The cells were centrifuged for 45 minutes at 1500 rpm. Lymphocytes were washed by double centrifugation for 10 minutes at 1500 rpm. The concentration of the cells was adjusted to 1×10^8 cells/ml [19].

The Romanovsky-Giemsa staining was used to count the number of spleen lymphocytes in the state of apoptosis among 100 cells in microscope field of view [1]. Apoptotic cells appeared as rounded or oval clusters of intensely eosinophilic cytoplasm with dense nuclear chromatin fragments. The nucleus may disintegrate into two or more fragments. These features allow distinguishing apoptotic cells from necrotic, which are characterized by the loss of plasma and nuclear membrane integrity and disintegration of cell organelles. The apoptotic vesicles are limited by plasma membrane and contain organelles. These preparations were studied with immersion system [18].

Kidneys were removed by autopsy, homogenized in a sterile mortar with a sand and 2 ml of sterile physiological solution with the subsequent seeding the 100 microliters of the samples to the solid growth media.

The total microbial number was determined on the beef-extract agar. *Staphylococcus* and *Streptococcus* spp. were determined in the selective agar medium for staphylococci and streptococci, respectively [17].

Statistical data processing was performed by the Lakin method (1980), using the Student criterion at the 0.05 level of significance [3].

Results and Discussion. In the literary data regarding the number of cells in a state of apoptosis in underbred mice under the condition of physiological norm are enough contradictory. Thus, for various organs and tissues the level of apoptotic cells varies from 0.5 to 69.0 % [2, 5]. Underbred mice were engaged in our research because of their extremely wide use in the experiments with infectious diseases reproduction [4]. Stably low values of apoptosis in underbred intact mice spleen cells, which varied in the range from 0 on the 7th day to 1.8 % on the 16th day, were obtained in our study (Fig. 1). In consideration of this fact the steady level of apoptosis, revealed for spleen lymphocytes of underbread intact mice can be noted.

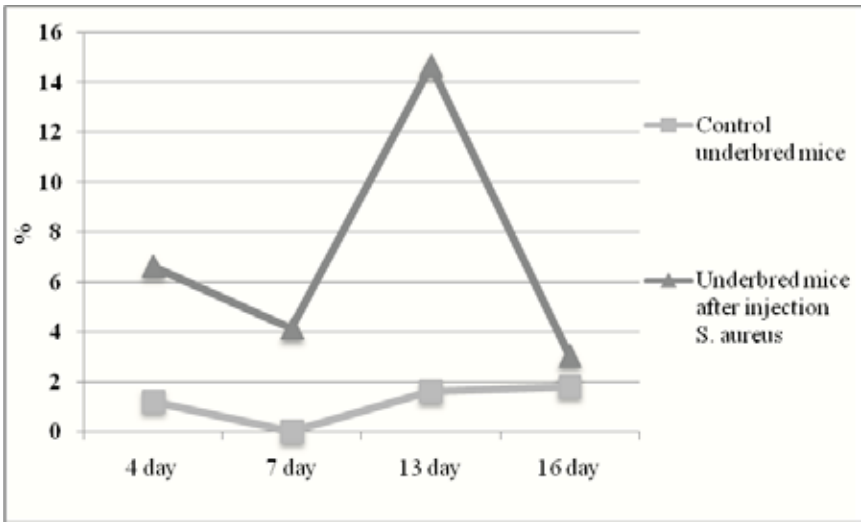

Fig. 1. Dynamics of changes of apoptotic cells number among spleen lymphocytes of intact and *S. aureus* infected underbred mice

Data obtained does not match the results of some of literature sources [8, 13], which showed a much higher number of cells in a state of apoptosis among the intact underbred mice splenocytes (5.4 – 7.9 %). However, a direct correlation between the level of apoptotic lymphocytes and infectious diseases was established in these studies [8, 13]: after complete elimination of the causative agent, the cells that participated in its destruction are eliminated by the apoptotic processes. So, including the low level of apoptosis among underbred intact mice splenocytes, we can assume that the experimental animals had no infectious diseases in the past.

A significant increase in the number of spleen lymphocytes in a state of apoptosis was observed after intravaginal injection of *S. aureus* cell suspension to underbred mice (Fig. 1). So, the number of apoptotic cells increased in 5.7 times – from 1.20 ± 0.30 % in the control to 6.66 ± 0.75 % ($P < 0.05$) on the 4th day. On the 7th day a quantity of these cells was equal to 4.16 ± 0.72 %, which was also higher than in the control group.

It is known [19] that the immune response development after intravaginal infection is slightly delayed in time (it was observed on the 7th – 9th days), thus the increasing of the apoptotic cells level on the 4th day may not be directly related to the injection of antigen (direct activation of Fas-receptors).

The highest level of apoptosis was revealed on the 13th day – the number of the cells in a state of apoptosis increased to 14.70 ± 1.13 % compared to 1.60 ± 0.31 % ($P < 0.05$). A significant reduction in the cell number in a state of apoptosis to the level of control (3.07 ± 0.44 %, in control group 1.80 ± 0.28 %; $P > 0.05$) was observed on the 16th day. This is probably due to the weakening of the *S. aureus* antigens stimulation in connection with the pathogen eliminating from the body [8].

The results of the lymphocyte's apoptosis study of the intact and *S. aureus* infected mice line BALB/c are shown on Fig. 2.

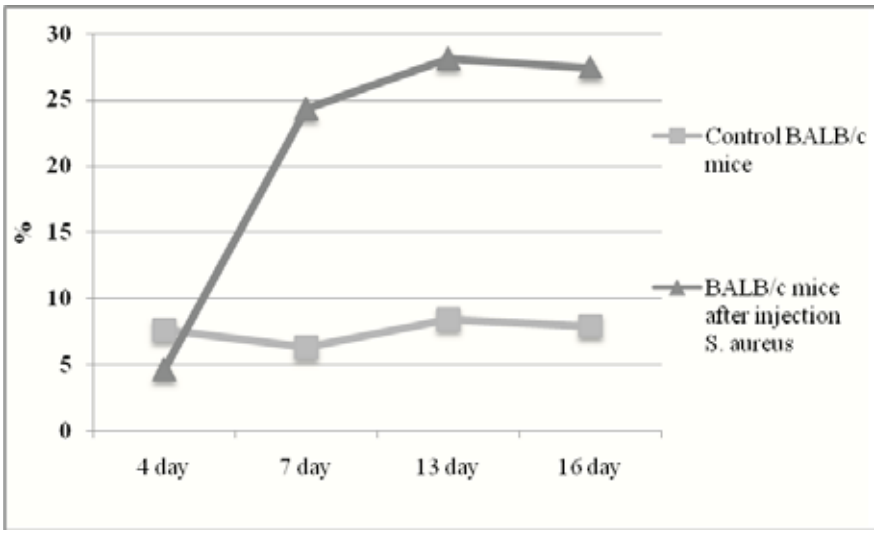

Fig. 2. Dynamics of changes of apoptotic cells number among spleen lymphocytes of intact and *S. aureus* infected BALB/c line mice

A slight fluctuation of the apoptotic cells number was indicated for the control group, the average was 7.54 ± 1.24 %. So, the number of the cells in a state of apoptosis among intact BALB/c line mice splenocytes is 4.18 times higher than the similar rates for underbred mice, that can indicate a feature of the immune system of this line mice, as it is well known [4] that the BALB/c line mice have specific congenital defects of some immunity branches.

The number of the spleen lymphocytes in the apoptotic condition did not change on the 4th day after BALB/c line mice being infected intravaginally by *S. aureus* (4.65 ± 0.72 %, in control group 7.57 ± 1.29 %; $P > 0.05$). It is known [14] that a certain period (no less than 5 days) is required for the direct cell apoptotic receptors stimulation by the antigen. However, a significant increase of apoptotic cells according to 24.32 ± 3.81; 28.12 ± 3.39 and 27.45 ± 4.01 % compared to 6.30 ± 0.92; 8.40 ± 1.14 and 7.90 ± 1.34 %, respectively, in the control ($P < 0.05$) was observed on the 7, 13 and 16th days.

The reduction of the immune cells with apoptosis characteristics is typical on the 7th – 9th day of the normal development of the immune response to the intravaginal injection of the pathogens, which is associated with an active elimination of the causative agent [2].

From the obtained data we can conclude the delay of the immune response in BALB/c line mice to *S. aureus*, since this group observed a consistently high level of spleen cells apoptosis from the moment of the infectious process development on the 7th day and up to the final 16th day of the experiment. We can assume that the defective immune response, typical of this line [4], led this case to the increase of the pathogens circulating period in the body.

The data obtained for ICR line mice was non-standard and did not match the materials, which were obtained for the previous groups (Fig. 3). The intact mice of this group were characterized by rather high average number of apoptotic cells – 11.68 ± 1.47 %.

A sharp decrease in the number of apoptotic lymphocytes was observed on the 4th and 7th days after intravaginal injection of *S. aureus* – according to 3.22 ± 0.43 and 7.14 ± 1.01 % compared to 11.03 ± 1.38 and 11.31 ± 1.26 % in the control ($P < 0.05$). On the 13th and 16th days their numbers have increased to 11.1 ± 1.24 and 11.53 ± 1.39 % against 12.50 ± 1.51 and 11.89 ± 11.32 %, respectively, in the control ($P < 0.05$).

A significant increase of aerobic and optionally anaerobic bacteria, indicating the spread of infection into all organs of the genitourinary system was found from the fourth day of the experiment, which was determined in the kidney's microbiological studies of the groups of mice with artificially reproduced infection (Table 1).

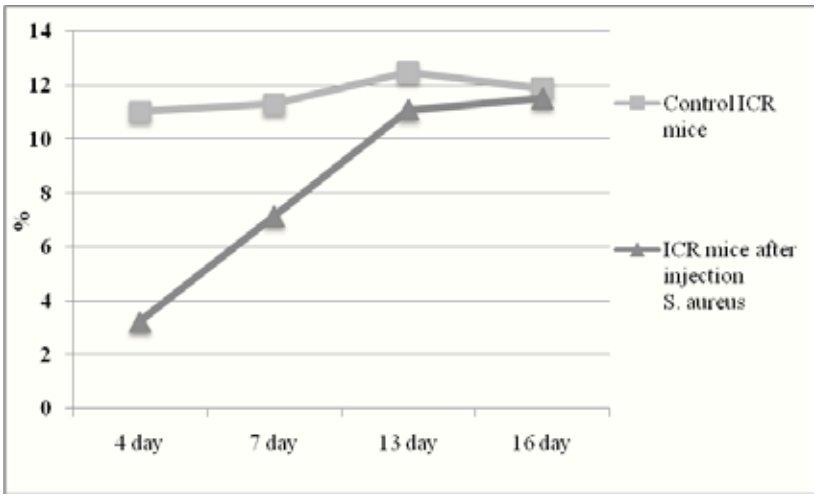

Fig. 3. Dynamics of apoptotic cells number among spleen lymphocytes of intact and *S. aureus* infected ICR line mice

The highest number of staphylococci colonies was observed in the kidneys on the fourth day of the underbred mice group study that can be completely logically explained by the injection of the congruous microorganism as an infection agent. However, the almost complete elimination of staphylococci with rapid development of streptococcal infection in the kidneys was observed further. This can be explained by «switching» of the immune system to the elimination of the main causative agent, accompanied by excessive growth of the attendant microorganisms [9].

Table 1

The kidney's microbiological research of different genetic lines and underbred mice

Mice	The day of study	CFU*/ml, Lg		
		Beef-extract agar	Selective agar medium for staphylococci	Selective agar medium for streptococci
Underbred	4	3.09 ± 0.11	3.49 ± 1.00	3.47 ± 0.98
	7	2.30 ± 0.34	0	0
	13	3.95 ± 0.18	0	4.34 ± 1.20
	16	4.07± 0.16	1.30 ± 0.02	4.64 ± 0.99
Line BALB/c	4	3.73 ± 0.01	0	3.95 ± 0.56
	7	3.69 ± 0.39	2.45 ± 0.45	3.06 ± 0.23
	13	3.74 ± 0.41	2.58 ± 0.98	3.95 ± 0.12
	16	3.72 ± 0.88	3.01 ± 0.56	3.92 ± 0.34
Line ICR	4	3.25 ± 0.91	3.59 ± 1.00	3.66 ± 0.76
	7	3.25 ± 0.35	3.19 ± 0.89	3.11 ± 1.20
	13	4.76 ± 0.45	4.65 ± 0.78	4.87 ± 0.98
	16	4.97 ± 0.34	4.76 ± 0.56	5.03 ± 1.10

* Colony forming unit

The excessive growths of aerobic and optionally anaerobic bacteria, including representatives of streptococcal group, were also detected for the mice of ICR and BALB/c lines. However, unlike the data from underbred mice, the rapid increase in the number of staphylococcal group of microorganisms, identified mostly as *S. aureus* was detected in the BALB/c and ICR lines. We can

make the hypothesis that the level of rates, noted in the microbiological study of kidney's suspension of infected BALB/c and ICR lines mice during the trial period was not maximum possible, and probably would still increase over some time.

Therefore, the research allows asserting that the intravaginal infecting of white underbred and BALB/c line mice resulted in a considerable increase of the number of spleen lymphocytes in a state of apoptosis. The highest number of apoptotic cells was on the 13th day after *S. aureus* injection.

This points to the infection transition to a generalized form with the subsequent development of completes (underbred mice) and delayed (line BALB/c) immune response. ICR line mice showed an atypical reactions to the intravaginal staphylococcal infection: the number of cells in a state of apoptosis decreased 3.4 times over a period from 4 to 7 day of the experiment as compared to the control. There are certain difficulties in interpretation of the results, as the increase in the number of apoptotic cells under the influence of staphylococcal antigens is a well-known fact [12].

The works that would give an explanation to the mechanisms of the phenomenon of apoptosis reduction after injecting the antigen to the mice of this line was not found in the literature. However, it is known that the feature of ICR line mice is the tendency to spontaneous tumor formation of different histogenesis. Given the existing data of spontaneous tumor formation mechanism, partly based on the reduction of the defective function cells apoptosis, there is likelihood that the development of these processes in ICR line mice is based on the defects of the immune system that causes the atypical response of immune cells to the pathogens entering [16]. It is possible that the violation of the apoptosis mechanisms is the cause of their susceptibility to spontanous tumors formation [7].

Conclusions

1. Intravaginal staphylococcal infection of underbred and BALB/c line mice was accompanied by apoptosis of lymphocytes and confirmed by an increasing number of splenocytes with morphological characteristics of apoptosis, which was the highest on the 13th day. However, the inhibition of apoptosis level was determined for the infected ICR line mice: the number of apoptotic cells among splenocytes decreased on the 4th – 7th day of the experiment, which is atypical of the flow of staphylococcal infection.

2. A high number of cells with morphological characteristics of apoptosis among intact ICR and BALB/c lines mice splenocytes in comparison with intact underbred mice is associated with a higher degree of kidney's microbial dissemination. The higher was the number of nonpathogenic microorganisms in the kidneys of intact ICR and BALB/c lines mice, the more apoptotic cells was indicated.

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АПОПТОЗ ЛІМФОЦИТІВ СЕЛЕЗІНКИ МИШЕЙ РІЗНИХ ПОПУЛЯЦІЙ ПРИ ЕКСПЕРИМЕНТАЛЬНІЙ СТАФІЛОКОКОВОЇ ІНФЕКЦІЇ УРОГЕНІТАЛЬНОГО ТРАКТУ

Р е з ю м е

У роботі наведено результати дослідження зміни рівня апоптозу серед клітин селезінки в динаміці розвитку інтравагінальної стафілококової інфекції на експериментальній моделі мишей різних генетичних ліній. Інтравагінальне інфікування мишей *Staphylococcus aureus* призводило до збільшення кількості клітин із морфологічними ознаками апоптозу в мишей безпородних та лінії BALB/c, тоді як у мишей лінії ICR їх кількість зменшувалась. Кількість клітин у стані апоптозу в мишей безпородних та лінії BALB/c супроводжувалась наявністю певного рівня мікроорганізмів, які висівались із нирок: чим вищою була кількість мікроорганізмів, тим вищим був рівень апоптозу спленоцитів.

К л ю ч о в і с л о в а: апоптоз, селезінка, *Staphylococcus aureus*, лінійні миші.

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АПОПТОЗ ЛИМФОЦИТОВ СЕЛЕЗЕНКИ МЫШЕЙ РАЗНЫХ ПОПУЛЯЦИЙ ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ СТАФИЛОККОКОВОЙ ИНФЕКЦИИ УРОГЕНИТАЛЬНОГО ТРАКТА

Р е з ю м е

В работе приведены результаты исследования изменения уровня апоптоза среди клеток селезенки в динамике развития интравагинальной стафилококковой инфекции на экспериментальной модели мышей различных генетических линий. Интравагинальное инфицирование мышей *Staphylococcus aureus* приводило к увеличению количества клеток с морфологическими признаками апоптоза у беспородных мышей и мышей линии BALB/c, тогда как у мышей линии ICR их количество уменшалось. Количество клеток в состоянии апоптоза у беспородних мышей и мышей линии BALB/c сопровождалось определенным уровнем микроорганизмов, которые высевались из почек: чем большим было количество микробов, тем выше оказался уровень апоптоза спленоцитов.

К л ю ч е в ы е с л о в а: апоптоз, селезенка, *Staphylococcus aureus*, линейные мыши.

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