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EFFECT OF RECOMBINANT HUMAN INTERLEUKIN-7 ON *Pseudomonas aeruginosa* WOUND INFECTION

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A wide range of interleukin-7 (IL-7) biological effects suggests that application of appropriate preparations in clinical practice will stimulate immunity in patients with lymphocytic exhaustion or autoimmune diseases. Studies are being conducted for IL-7 based preparations aimed at restoration of the immune system of patients with immunodeficiency of different origins. Pseudomonas aeruginosa is an important pathogen, which causes nosocomial infections in hospitalized patients. Infection factors, affecting the immune status of the host, play a key role. A promising and relevant scientific endeavor is the study of the effect of recombinant IL-7 (rIL-7) as an adjunct therapy in wound infections caused by P. aeruginosa. The aim of this study was to evaluate the effectiveness of rIL-7 use in P. aeruginosa wound infection in mice. The experiments were conducted using a standardized rIL-7 preparation, P. aeruginosa strain and 20 white non-inbred mice. The preparation of rIL-7 after all stages of purification was characterized by the content of ballast proteins and impurities via electrophoresis in a polyacrylamide gel in reducing conditions, and its biological activity was evaluated in the MTT test by means of proliferation of peripheral blood mononuclear cells. In the mice, the fur was removed, the neck nape was intentionally injured and P. aeruginosa bacteria were injected into the wound of each animal (0.1 ml of suspension with a bacterial cell concentration of 0.08×10^9 cells/ml). Starting from the 2nd day, bacterial examination of the wound material was carried out daily. Starting from the 3rd day, the mice (experimental group, n = 10) were intraperitoneally administered 5 μg (0.1 ml) of the rIL-7 preparation. In the control group of animals (n = 10), the rIL-7 preparation was not administered. In 80% of experimental animals (administered the rIL-7 preparation), the healing of wounds and elimination of the pathogen of purulent inflammatory infection P. aeruginosa occurred on the 7th day. On the 9th day from the beginning of wound infection, wound healing and elimination of P. aeruginosa occurred in all experimental mice. In 60% of mice from the control group (did not receive treatment with rIL-7), wound healing and the elimination of P. aeruginosa occurred on the 9th day. Wound healing and elimination of P. aeruginosa in all mice of the control group occurred on the 14th day. Thus, in mice treated with rIL-7, wound healing and elimination of the pathogen occurred 5 days earlier than in mice from the control group (without rIL-7 treatment). Subsequent studies may be aimed at developing protocols for the treatment of wound infections using an rIL-7 preparation in patients with a compromised immune system Therefore, rIL-7 is a promising preparation for the treatment of complex wound infections.

K e y w o r d s: recombinant human interleukin-7, Pseudomonas aeruginosa, wound infection, therapy.

Interleukin-7 (IL-7) is a central cytokine of the immune system, which plays an important role in the modulation of T- and B-cell development and T-cell homeostasis [1, 2]. The potential and wide range of its effects suggest that IL-7 administration will stimulate immunity in patients with lymphocytic exhaustion and autoimmune diseases. Currently, active IL-7 studies are being conducted as a means to restore the immune system against the background of immunodeficient states of various origins. IL-7 therapeutic activity is shown in solid tumors, bacterial and viral infections [4-8].

Recently, there has been a tendency for the emergence of many *P. aeruginosa* strains with resistance to a wide range of antibiotics, which requires the development of alternative therapeutic approaches [9-13]. This is primarily due to intrahospital infection by these microorganisms, as the frequency of their selection increases in proportion to the length of time a patient stays in the hospital. As a rule, *P. aeruginosa* infection develops in patients with reduced natural resistance (e.g., with burns, oncological diseases, use of immunosuppressants). This pathogen is characterized by resistance to many antimicrobial substances, which causes a high mortality from the sepsis that develops [14-16]. The resistance of *P. aeruginosa* strains to disinfectants prevents their elimination from clinics [17, 18].

This problem is becoming even more relevant for military field surgery [19, 20]. For Ukraine as a region of military action, the increase in the number of contingents of patients undergoing various surgical interventions and manipulations, and the use of new invasive methods of diagnosis and treatment are typical. All of these circumstances determine an increase in intra-hospital infections caused by pathogenic and opportunistic microorganisms, including *P. aeruginosa*. It should be noted that in the case of the *P. aeruginosa* infection, factors that affect the state of the macro-organism immune system play a special role [21].

Given available literature data, the study of the effect of recombinant IL-7 (rIL-7) as an adjunct therapy in wound infections, in particular due to *P. aeruginosa*, is a promising and relevant scientific endeavor. It should be noted that our analysis of scientific publications of recent years did not reveal work on the study of the effects of IL-7 preparations on the course of wound infections. Thus, this lack of scientific literature determined the innovative approach of our work.

The aim of our study was to evaluate the effectiveness of using an rIL-7 preparation in wound infections in mice caused by *P. aeruginosa*.

Materials and Methods

Laboratory animals. Twenty white non-inbred (non-breeding) mice weighing 14-18 g were used in the studies (males at the age of 3 weeks) (Gromashevsky Institute of Epidemiology and Infectious Diseases of the National Academy of Science of Ukraine, Kyiv, Ukraine). Studies on animals were carried out in compliance with bioethical norms [23], including the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986). The permission for animal work was issued by the ethics committee of Gromashevsky Institute of Epidemiology and Infectious Diseases of the National Academy of Science of Ukraine.

rIL-7 preparation and its administration. Recombinant human interleukin-7 was provided by UA Pro-Pharma LLC (Kyiv, Ukraine). The preparation was obtained using recombinant DNA technology in an expression system based on the *E. coli* BL21 (DE3) strain and the plasmid vector pACYC184. The cytokine was purified from impurities via usage of gel filtration on Sephadex G-25 and ion-exchange chromatography on Q and SP Sepharose (Merck, USA). Control of purity of the preparation was performed via polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE), and biological activity was determined by means of mononuclear peripheral blood cells (MPBC) from healthy donors (cell proliferation evaluation was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide – MTT-test) [22]. In this study, rIL-7 preparations at concentrations of 20 μg/ml were used.

Each mouse in the experimental group (n = 10) starting from the 3rd day of the experiment was injected intraperitoneally with 5 μg (0.1 ml) of rIL-7 preparation; administration lasted for 7 days. Thus, each animal in the experimental group received 35 μg of rIL-7. In the control group of animals (n = 10), the rIL-7 preparation was not administered (only saline was administered).

P. aeruginosa strain characteristics. This bacterial strain is characterized and stored in the museum of human pathogenic microorganisms of the Gromashevsky Institute of Epidemiology and Infectious Diseases of the National Academy of Science of Ukraine (Kyiv, Ukraine). The microorganism

has the following characteristics: it is mobile; has pigments pyocyanin and fluorescein; synthesizes trimethylamine; is oxidase- and catalase-positive and exhibits hemolysis on blood agar; grows at a temperature of 42 °C, but not at 50 °C; converts nitrates to nitrites, has a positive test for arginine dihydrolase, and negative test for lysine- and ornithine decarboxylase; and forms an acid from glucose. On Endo's medium and meat-peptone agar (MPA) it forms wavy colonies (resembling "daisy flowers"). It is resistant to the following antibiotics: ciprofloxacin, ceftazidime, amikacin, cefepime, meropenem; but is sensitive to colistin. The strain is also resistant to the action of many disinfectants: Septamine (dichloroisocyanuric acid sodium salt, trichloroisocyanuric acid), Saneedes (trichloroisocyanuric acid, isocyanuric acid), Maxisan (quaternary ammonium salts mixture), Dysmoson (magnesium monoperoxy phthalate hexahydrate), Santifect (*p*-alkyl dimethylbenzyl ammonium chloride, *p*-alkyl dimethyl ethylbenzyl ammonium chloride), Entaktyv (benzalkonium chloride), Dezekon (didecyldimethylammonium chloride, aminopropyl dodecylpropane thiamine).

Modeling of wound infection in mice. The study involved 20 mice. The fur on the napes of the animals was removed and wounds the size of 1-1.5 cm were made with a safety razor blade. The *P. aeruginosa* culture was grown for 24 h on MPA at a temperature of 37 ± 1 °C. From the 24-hour culture, bacterial inoculum was made, the turbidity of which corresponded to 10 units at the standard sample of turbidity (OSZ 42-28-85-01 P) (corresponding to a concentration of bacterial cells of 0.08×10^9 cells/ml). 0.1 ml of a bacterial suspension was injected into the wound of each mouse.

Microbiological tests. During the entire study the monitoring of the manifestation of wound infection and the size of the wound were performed via visual observation with photo fixation. The method of serial dilutions was used for quantitative microbiological assessment. The primary dilution 1:10 was prepared as follows. The wound drainage was introduced into a test tube with a sterile isotonic solution of sodium chloride. Further dilution (1:10) was transferred to 0.1 ml of inoculum into a test tube of 9.9 ml of sterile isotonic sodium chloride solution. Thus, the dilutions were prepared up to 10^{-7}. Dilutions of wound material was seeded on Petri dishes with MPA (0.1 ml of wound material) with subsequent uniform rubbing into the surface of the medium using a spatula to obtain the growth of isolated colonies (for each dilution). Petri dishes were incubated at 37 ± 1 °C for 24 h. The next day, in the presence of growth from certain dilutions, the number of grown colonies on agar was counted. The number of microorganisms in 1 cm^2 (N) was determined via the following formula:

$$N = n \times a \times b,$$

where *n* is the number of colonies grown on the Petri dish; a is the coefficient of seeding dose (a = 10, when seeding dose is 0.1 ml); *b* is the wound material dilution degree.

Results and Discussion

Characterization of rIL-7 preparation. The preparation of rIL-7 after all stages of purification was characterized by the content of ballast proteins and impurities with SDS-PAGE (Fig. 1). Its biological activity was evaluated by the MTT-test and MPBC. Biological activity was evaluated in comparison to the standard sample of rIL-7 (calibrator) produced by PeproTech (Rocky Hill, NJ, USA), which allowed the calculated content of the test cytokine in the preparation sample. Validation of this method as a method of quantitative determination was carried out previously [22].

Modeling of *P. aeruginosa* infection in wounds. As described above, all mice were infected with *P. aeruginosa* bacteria. At the same time, the clinical signs of wound infection were the same for all animals. The following clinical manifestations of *P. aeruginosa* infection were recorded: hyperemia of the tissues around the wound, limited edema, a sufficient difference in the edges of the wound, and purulent discharge from the incision.

On the 2nd day, wound culture of the detachable material from wounds was taken in order to isolate the pathogen and determine its amount. Seeding was carried out in 10 ml of sterile isotonic sodium chloride solution. From each tube was made hanging on cups of MPA in an amount of 0.1 ml, followed by rubbing into the surface of the medium using a spatula. The cups were incubated at 37 ± 1 °C for 24 h. The number of grown colonies on agar was counted the next day. For the 3rd day from the beginning of the wound infection, the number of *P. aeruginosa* microbial cells per 1 cm^2 of the wound surface ranged from 1.0×10^2 to 1.0×10^6 cells/cm^2.

During the 2nd day, two experimental groups of animals were formed: experimental and control, with 10 mice in each. Starting from the 3rd day after the beginning of the experiment, each experimental mouse received an intraperitoneal injection of 5 μg

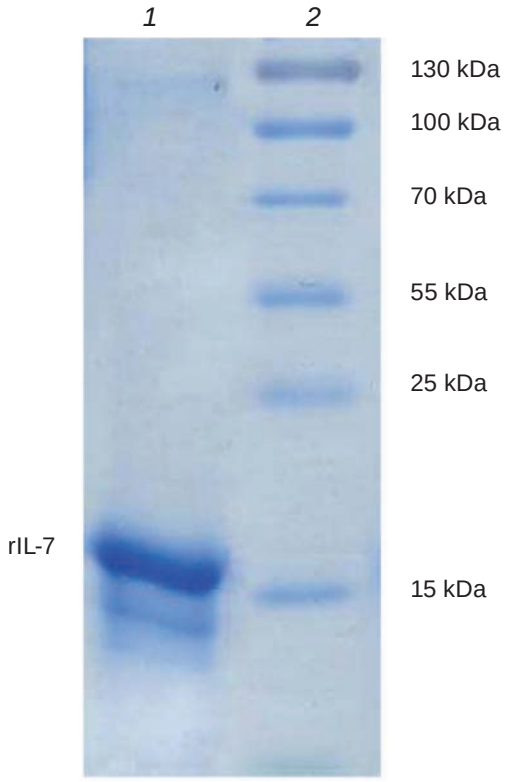

Fig. 1. Electrophoregram of the rIL-7 preparation: Lane 1 – rIL-7 preparation; Lane 2 – molecular weight markers (130, 100, 70, 55, 25, 15 kDa)

of the rIL-7 (0.1 ml) per day. In the control group of animals, the rIL-7 preparation was not administered.

On the 5th day after the beginning of the experiment (or the 3rd day after the administration of the rIL-7 preparation), wound cultures were again taken to determine the qualitative and quantitative findings of *P. aeruginosa* in the material separated from the wound. According to national recommendations, the fact of isolating the microorganism from the wound is evidence of its etiological significance in the infectious purulent-inflammatory process [24].

The obtained data indicated that on the 5th day after the beginning of the experiment, the number of *P. aeruginosa* microbial cells per 1 cm^2 of wound surface varied from 1.0×10^4 cells/cm^2 (the lowest value) to 5.0×10^5 cells/cm^2 (the highest value) in the experimental group of mice receiving rIL-7 intraperitoneally. The number of *P. aeruginosa* microbial cells per 1 cm^2 of the wound surface of the mice in the control group (which did not receive treatment with the cytokine preparation) varied from 3.0×10^3 cells/cm^2 (the lowest value) to 5.0×10^6 cells/cm^2 (the highest value) – one order of magnitude greater than the maximum value in the experimental group of mice. External manifestations of wound infections in the mice of both groups on the 5th day after infection with *P. aeruginosa* was the same as on the 3rd day, but they were more pronounced.

On the 7th day from the start of the experiment there was a marked tendency toward wound healing (dryness, formation of a scab) in mice treated with the rIL-7 preparation. Seed cultures were marked with the absence of pathogen, which showed that *P. aeruginosa* completely disappeared from the surface of the wound of the mice of the experimental group (with rIL-7 administration).

There was a completely different situation on the 7th day in the control group of mice. Only one experimental animal was characterized with the absence of bacteria in the wound. In other mice the number of *P. aeruginosa* microbial cells per 1 cm^2 of wound surface varied from 1.0×10^3 cells/cm^2 to 1.0×10^5 cells/cm^2. The following signs of wound infection in control animals were still present: hyperemia and scabs formation.

On the 9th day there was complete elimination of the *P. aeruginosa* causative agent in mice treated with the rIL-7 preparation, with externally well-marked healing wounds as evidenced by wound culture taken on the 9th day.

On the 9th day there was a tendency toward healing and elimination of the pathogen in half of the mice from the control group. The rest of the mice were still characterized by infection of the wound, which was confirmed by microbiological cultures (the amount of the *P. aeruginosa* agent remained at a sufficiently high level – 1.0×10^5 cells/cm^2 of the wound surface).

The 12th day was marked with almost complete healing of the wound of the experimental group of mice up to the beginning of restoration of the fur cover. *P. aeruginosa* from the wounds was not found.

In half of the mice from the control group the pathogen was not found in the wounds on the 12th day of the experiment; there was healing of the wound. The other mice still had signs of infection of the wound. The pathogen *P. aeruginosa* was excreted in significantly smaller numbers – $(3.0 \text{ to } 5.0)\times10^3$ microbial cells per 1 cm^2 of the wound surface.

Complete elimination of the pathogen from the wound surface in the control group of mice occurred on the 14th day after the wound was infected by *P. aeruginosa*. The recovery dynamics of mice with wound infection by *P. aeruginosa* in experimental and control groups of animals is shown in Fig. 2.

Fig. 2. Dynamics of wound infection by P. aeruginosa in experimental and control mice

Most researchers attribute *P. aeruginosa* to conditional pathogens that can exhibit their invasive and toxic properties in extreme situations [14-18]. The frequency of purulent-inflammatory complications of *P. aeruginosa* etiology can also be provoked by the fact that this agent is very unpretentious to

nutrients in conditions of the hospital; it has long been stored in the environment; it is highly resistant to antibiotics and antiseptics and produces a large number of various extracellular toxic substances that can suppress macroorganism natural resistance [21]. Pathogenicity factors include ones which are toxic to neutrophils and have endotoxin-like properties: surface cell components (mucus polysaccharide, mucoid polysaccharide, lipopolysaccharide O-antigen, lipid A). Mucus polysaccharide of *P. aeruginosa* has an anti-phagocytic effect and suppresses local pulmonary immunity. Pathogenicity factors also include the following extracellular products: proteases, exotoxin A, phospholipase C – lecithinase, suppressing complement-dependent mechanisms of protection and possessing toxicity for macrophages [21, 25]. Thus, the state of the immune system has an important and sometimes crucial significance for predicting the consequences of wound infections caused by *P. aeruginosa*.

Several examples of the use of cytokines, in particular interleukin-2 (IL-2) and interferon-alpha 2b, in the treatment of wound infections are described in the literature [7, 8]. For example, the administration of IL-2 resulted in an increase in the number of lymphocytes and macrophages in the wound, accelerating the changes in the stages of the wound process and increasing the activity of phagocytic cells in the wound, which contributed to faster wound healing.

IL-7, the cytokine we investigated, is the first type of short-chain cytokines in the family of hematopoietins [26, 27]. It is known that the main sources of IL-7 formation in the human body are non-hemopoietic cells of the stroma of the bone marrow, thymus, and lymphoid organs. One of the first biological properties of IL-7 that was detected is its ability to activate precursors of B cells, as well as immature and mature T-cells [26, 27]. At present, the key role of IL-7 in T- and B-lymphopoiesis has been proved [28]. Among the important effects of this cytokine, its role as modulator of low-affinity peptide-induced T-cell proliferation should be noted. This property is considered as an important mechanism of homeostatic regulation of T-cell populations. Data prove an increase in the level of IL-7 in plasma and lymph in response to the depletion of the T-cell lymphocytes in favor of this mechanism [29]. Among other things, the participation of IL-7 in the development of dendritic cells, natural killers and lymphoid tissue inducer cells has been proved [1]. There is an interesting data regarding a deficit of IL-7 in septic patients [30]: septic patients showed the lowest levels of IL-7. Decrease of a number of T cell populations in patients with sepsis was demonstrated. The authors of this work proved a crucial role of IL-7 as a factor of development of γδ T cells, as well as survival in sepsis, IL-7 association with sepsis severity, evolution of organ failure and even death.

Therefore, our results are in good agreement with the available literature data on the pathogenesis of *P. aeruginosa* infection, as well as on the immunomodulatory properties of IL-7. In our opinion, the obtained results are an important prerequisite for the development of protocols for the integrated treatment of wound infections, especially in patients with a compromised immune system, as well as for the development of finished dosage forms based on rIL-7.

In summary, in 80% of experimental animals which were administered intraperitoneal rIL-7, the healing of wounds and elimination of the pathogen of purulent inflammatory infection *P. aeruginosa* occurred on the 7th day. On the 9th day from the beginning of wound infection, wound healing and elimination of *P. aeruginosa* occurred in all experimental mice. In 60% of mice from the control group (did not receive treatment with rIL-7), wound healing and the elimination of *P. aeruginosa* occurred on the 9th day. Wound healing and elimination of *P. aeruginosa* in all mice of the control group occurred on the 14th day. Thus, wound healing and pathogen elimination occurred 5 days earlier in mice treated with rIL-7 than in mice from the control group (without rIL-7 treatment). Therefore, rIL-7 is a promising preparation for the treatment of complex wound infections. Subsequent studies may be aimed at developing protocols for the treatment of wound infections using an rIL-7 preparation in patients with a compromised immune system.

ВПЛИВ РЕКОМБІНАНТНОГО ІНТЕРЛЕЙКІНУ-7 ЛЮДИНИ НА РАНОВУ ІНФЕКЦІЮ *Pseudomonas aeruginosa*

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Широкий спектр біологічних ефектів дозволяє припустити, що застосування в клінічній практиці інтерлейкіну-7 людини (ІЛ-7) дозволить стимулювати імунітет у пацієнтів з лімфоцитарним виснаженням, автоімунними захворюваннями тощо. Наразі ведуться дослідження ІЛ-7 як засобу для відновлення імунної системи на фоні імунодефіцитних станів різного походження. Вивчення впливу препарату рекомбінантного ІЛ-7 (рІЛ-7) як супутньої терапії ранових інфекцій, спричинених *Pseudomonas aeruginosa*, є перспективним науковим завданням. Метою роботи було оцінити ефективність використання рІЛ-7 у разі ранової інфекції *P. aeruginosa* в мишей. Дослідження проводили з використанням: стандартизованого препарату рІЛ-7, штаму *P. aeruginosa*, білих неінбредних (безпородних) мишей. Препарат рІЛ-7 після всіх етапів очистки був охарактеризований щодо вмісту баластних протеїнів та домішок за допомогою електрофорезу в поліакриламідному гелі в редукуючих умовах, а його біологічну активність було оцінено у МТТ-тесті на мононуклеарних клітинах периферичної крові. Шерсть на загривку тварин вистригли та наносили поранення, і в рану кожної тварини вводили *P. aeruginosa* (0,1 мл суспензії із вмістом бактеріальних клітин $0{,}08\times10^9$ клітин/мл). Починаючи з 2-го дня інфікування проводили бактеріальне дослідження ранового матеріалу. З 3-ї доби мишам (експериментальна група, $n = 10$) внутрішньочеревно вводили 5 мкг (0,1 мл) препарату рІЛ-7. Контрольній групі тварин ($n = 10$) препарат рІЛ-7 не вводили. У 80% тварин (із введенням препарату рІЛ-7) загоєння ран і елімінація збудника гнійно запальної інфекції *P. aeruginosa* відбувалося на 7-му добу. На 9-ту добу від початку зараження рани відбувалося загоєння ран і елімінація *P. aeruginosa* у всіх дослідних мишей. У 60% мишей з контрольної групи, що не отримували лікування препаратом рІЛ-7, загоєння ран і елімінація збудника *P. aeruginosa* відбувалася на дев'яту добу. Загоєння ран і елімінація *P. aeruginosa* у всіх мишей контрольної групи відбувалася на 14-ту добу. Тобто в мишей, що отримували лікування препаратом рІЛ-7, загоєння ран і елімінація збудника наставала на 5 днів раніше, ніж у мишей з контрольної групи. Таким чином, рІЛ-7 є перспективним препаратом для комплексної терапії ранових інфекцій.

Ключові слова: рекомбінантний інтерлейкін-7 людини, *Pseudomonas aeruginosa*, ранова інфекція, терапія.

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