UDC: 612.017.1.579.861.097.2

MODELING THE IMMUNOBIOLOGICAL DRUG COMBINATIONS WITH ANTIVIRAL AND IMMUNOMODULATORY EFFECT TO ENHANCE PROTECTIVE IMMUNITY

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The article discusses the immunobiological activity of human granulocyte colony stimulating factor (G-CSF) (Filsm), recombinant interferon α-2b (Laferobion), Herpevir (international name Acyclovir) and nano-sized cell wall biopolymer of Staphylococcus aureus. We analyzed the effect of the combinations of these drugs on the development of protective immunity and conducted optimization of the drug production process to achieve antiviral, immunomodulatory and immuneregulating effects. Application of the staphylococcal adherence protein (EAP) biopolymer in combination with aforementioned drugs resulted in synergistic immunomodulatory activity, production of protective cytokines, and enhancement of protective immunity. Thus, a nano-sized biopolymer can be used to potentiate other drugs with similar properties.

Keywords: immunobiological drugs, G-CSF, Filstim, Laferobion, Hepervir, Acyclovir, *Staphylococcus aureus*, biopolimer EAP, immunocorrection, protective immunity.

ОДЕРЖАННЯ МОДЕЛЕЙ КОМБІНОВАНИХ ІМУНОБІОЛОГІЧНИХ ПРЕПАРАТІВ, ЩО ВОЛОДІЮТЬ ПРОТИВІРУСНОЮ ТА ІМУНОКОРЕГУЮЧОЮ ДІЄЮ ДЛЯ ЗАБЕЗПЕЧЕННЯ ПРОТЕКТИВНОГО ІМУНІТЕТУ

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У статті розглянуто імунобіологічна активність людського гранулоцитарного колоніє стимулюючого фактора Г-КСФ (Філстим), інтерферону α-2b рекомбінантного (Лаферобіон), Герпевіру (міжнародна назва Ацикловір) та нанорозмірного біополімеру клітинної стінки Staphylococcus aureus. Також проаналізовано впливи моделей комбінованих імунобіологічних препаратів для забезпечення протективного імунітету та оптимізацію процесу виробництва препарату, що володіє противірусною, імуномодулюючою та імунокорегуючою дією. Показано, що при комбінованому застосуванні досліджуваного біополімера стафілокока (білку-адгезину ЕАР) та фармацевтичних препаратів спостерігали синергізм дії по імуномодулюючим властивостями, продукції захисних цитокінів та забезпеченні протективного імунітету. Отже отриманий нанорозмірний біополімер може бути використаний для моделювання біофармацевтичних препаратів з подібними властивостями.

Ключові слова: імунобіологічні препарати, Г-КСФ, Філстим, Лаферобіон, Гепревір, Ацикловір, *Staphylococcus aureus*, біополімер ЕАР, імунокорекція, протективний імунітет.

In the current study the combined action of the following drugs was examined: Filstim, Laferobion (LLC «Pharmaceutical plant «Biofarma», Bila Tserkva, Ukraine), Gerpevir (PJSC Kyivmedpreparat, Kyiv, Ukraine), Acyclovir (Pharmaceutacal Firm «Darnitsa», Kyiv, Ukraine) and the nano-sized cell wall biopolymer of *Staphylococcus*- EAP (p70) [4].

Febrile neutropenia, which occurs as a result of the use of cytostatics for cancer treatment, is the most common treatment complication, which results in cumulative decrease in chemotherapy doses and reduces the therapeutic effect [1]. The stage and duration of developing neutropenia after chemotherapy, largely determines the risk of life-threatening infectious complications. The introduction of a granulocyte colony stimulating factor (G-CSF) into the clinical practice made it possible to reduce the duration of neutropenia and minimize the negative effects of myelosuppressive cancer therapy. Today, many studies have proved the efficiency of G-CSF for treatment of cancer patients [2, 18].

Filgrastim is a highly purified non-glycosylated polypeptide (175 amino acids). It is produced by a genetically modified culture of Escherichia coli, which contains

human G-CSF gene. Human G-CSF regulates the formation of functionally active neutrophil granulocytes and their entry into the blood from the bone marrow. Like other hematopoietic growth factors, G-CSF can stimulate endothelial cells in vitro, which can stimulate the growth of myeloid cells, including malignant cells in vitro [18,21]. Similar effects have observed for non-myeloid cells in vitro. Filstim containing recombinant G-CSF significantly increases the number of neutrophil granulocytes in the peripheral blood during the first 24 hours after injection and simultaneously causes an increase in the number of monocytes. The increase in the number of neutrophil granulocytes and their functional characteristics are dose-dependent [23]. The use of Filstim drugs significantly reduces the frequency and duration of neutropenia in patients after chemotherapy with cytostatics, myeloablative therapy with subsequent bone marrow transplantation [24]. Patients receiving the drug rarely need hospitalization, spend less time in the hospital, need lower doses of antibiotics compared with patients who received only cytotoxic therapy. The use of Filstim (both primary and after chemotherapy) activates peripheral blood precursor cells (PBPS) [19]. In children and adults with severe chronic neutropenia (severe congenital, periodic and malignant neutropenia), the drug stably increases the number of peripheral blood neutrophils and reduces the incidence of infectious complications. After the treatment is completed, the amount of granulocytes in the peripheral blood decreases by 50% within 1-2 days and returns to normal within 1-7 days [20, 22].

Inducers of endogenous interferon (interferonogens) are widely represented in the modern pharmacological market. These drugs differ in chemical structure, but they all have the common property - the ability to stimulate the production of interferons by epithelial cells, lymphocytes and macrophages of the human body. The advantage of such therapy is the absence of severe systemic effects observed with the use of interferons, as well as the preservation of endogenous interferonogenesis, which is suppressed by long-term use of high doses of recombinant interferon preparations. In this regard, such drugs may be recommended for long-term therapy, this is often required for treatment of various viral infections.

Recombinant Interferon α-2b belongs to a group of endogenous low molecular weight proteins, has a pronounced antiviral, antiproliferating and immunomodulatory effect. The complex composition of Laferobion causes a number of new effects: in combination with tocopherol acetate and ascorbic acid, the antiviral activity of the interferon α-2b recombinant increases 10-14 times, its immunomodulatory effect on T- and B-lymphocytes increases, and the IgE content is normalized. There are no antibodies that neutralize the antiviral activity of recombinant interferon-α-2b, even when it is used up to 2 years, in addition, the engogenous production of interferons is normalized [13].

The active ingredient of the drug, interferon α-2b, is a highly purified human recombinant protein that consists of 165 amino acids and with a molecular weight of 19 kDa. The drug is derived from the E. coli clone by plasmid hybridization with the human leukocyte gene that codes for the synthesis of interferon. The antiviral effect of the drug is achieved due to its interaction with specific membrane receptors, the induction of mRNA synthesis and, eventually, the synthesis of proteins that interfere with the normal reproduction of the virus. Immunomodulatory activity of the drug is associated with activation of phagocytosis, which stimulates the formation of antibodies and lymphokines. The drug also has antiproliferative effect on cells of malignant neoplasms [13, 14].

In the renaturation process of interferon molecules, formation of incorrect intra- and intermolecular bonds can be observed. This results in partial formation of irregular monomeric forms of recombinant interferon, the conformation of which is different from that of natural interferon, as well as the appearance of oligomeric structures which are not present in natural interferon. The N-terminal amino acid residue of interferon α-2b is cysteine, which is bound by a disulfide bond with cysteine at position of 98 of the recombinant protein. Also, in some molecules of recombinant interferon alpha-2b secreted from Escherichia coli, an additional residue of methionine (formylmethionine) remains at the N-terminus, which makes interferon alfa-2b a modified protein. Excessive N-terminal methionine can affect the stability and immunogenicity of

proteins, and in most cases, it leads to the loss of their biological activity. Pharmacokinetic characteristics of the recombinant interferon alfa-2b demonstrate that its levels are not maintained at the constant level in plasma, this partially explains its transient therapeutic effect. Most of the known modifications of interferon preparations aims either to increase the physical size (hydrodynamic volume) or charge (negative) of the recombinant molecules, in order to slow down the process of their filtration from the plasma through the pores of the renal tubules [15]. Combination of interferon α-2b with polyethylene glycols (PEG), interferon-α-2b PEG, allows to maintain prolonged plasma glucose levels and has improved pharmacokinetic profile [16, 17].

As a result of modern studies on the binding of IFN to cells, it has been found that IFN specifically binds to cells that are sensitive to this type of interferon. Insensitive cells, such as resistant cells of the respective animal species, and naturally-insensitive cells of other species, do not specifically bind to IFF. This is due not only to the specific features of the IFN molecules but also to the structural differences between cellular receptors. In order to strengthen IFN binding, we tried to modify the interferonogenesis by using a biofilm of the cell wall of Staphylococcus [4].

Herpevir (Acyclovir - intl. name) is an antiviral drug, it is a synthetic analogue of purine nucleoside that has high in vitro and in vivo activity against the herpes simplex virus I and II, herpes zoster, Epstein-Barr virus and cytomegalovirus [13]. The drug has minimal toxicity to the host cells. Once intracellular, acyclovir is phosphorylated with a formation of the active compound - acyclovir triphosphate. The first stage of this process depends on the presence of the virus-coded thymidine kinase. Acyclovir triphosphate acts as an inhibitor and substrate for viral DNA polymerase, preventing further synthesis of viral DNA without affecting conventional cellular processes. The inhibitory activity of acyclovir against the above viruses is highly selective [16]. The thymidine kinase enzyme in a normal non-infected cell does not use acyclovir as a substrate, therefore the toxic effect on host cells is minimal. However, thymidine kinase, encoded in simplex herpes viruses, chicken pox viruses, herpes zoster and Epstein-Barr virus, converts acyclovir to acyclovir monophosphate, an

analogue of nucleoside, which is then converted sequentially to diphosphate and triphosphate with the help of cellular enzymes. Following the incorporation of acyclovir into the viral DNA, triphosphate interacts with the viral DNA polymerase, which results in the termination of the synthesis of the viral DNA. In prolonged and repeated treatment courses of severe immunocompromised patients, the sensitivity of individual strains of the virus may be reduced, which is not always consistent with acyclovir treatment [17]. The majority of clinical cases of insensitivity are associated with a deficiency of viral thymidine kinase, but there are reports of damage to viral thymidine kinase and DNA. In vitro interaction of individual herpes simplex viruses with acyclovir may also lead to the formation of less sensitive strains. The interdependence between the sensitivity of individual herpes simplex viruses in vitro and the clinical outcome of treatment with acyclovir has not been fully understood [13] and may be related to the induction of interferon, TNF and other cytokines - the regulators of the immunopoiesis.

The aim of the study: To investigate the immunobiological activity of granulocyte colony stimulating factor (Filstim) and biopolymer of cell wall of staphylococcus in mice. The literature about prevention of febrile neutropenia and reduction of its duration was analyzed. The phagocytic activity of murine macrophages and the pharmacological activity of the Filstim preparation on the model of febrile neutropenia was investigated.

To determine the immunobiological properties and immunomodulating effects of Laferobion combined with a highly purified, biochemically characterized cell wall 70 kDa biopolymer (EAP) of the *Staphylococcus aureus* to obtain a combined immunobiological drug model for the protective immunity. The antiviral activity of the studied drugs was evaluated as described [8, 9].

To explore the combined effects of the synthetic drug Gerpevir or Acyclovir with a biopolymer of natural origin. The immunobiological activity and the effects of the combinatory drug on the synthesis of endogenous interferon and tumor necrosis factor were examined.

Materials and methods.

The highly purified biopolymer of the cell wall of *Staphylococcus* (protein-adhesin EAP (mW 70 kDa) was obtained by a combination of several methods using ion exchange chromatography and gel filtration. The biochemical properties of EAP were described by us earlier [4].

Phagocytic cells and lymphocytes were activated by subcutaneous injection of a *Staphylococcus aureus* (EAP) cell wall biopolymer at a dose of 1 mg / kg. Experimental leukopenia in male C57BL/6 mice was simulated by single i.p. injection of cyclophosphamide (Sandoz Pharmaceuticals d.d. Slovenia) at a dose of 250 mg/kg. The drug Filtism (LLC «PP Biopharma», Bila Tserkva, Ukraine, a solution for injection of 1 ml in a vial) was injected once daily by subcutaneously at a dose of 1 mg/kg in 24 hours after the injection of cyclophosphamide, or after 24 hours after injection of 0.9% solution of sodium chloride. On day 7 after the introduction of the study blood cells differential counts were examined in blood smears. After euthanasia, the bone marrow cells were isolated from the femur, and the total number of cells was calculated, and in the bone marrow smear - myelogram, leukocyte to erythrocyte ratio, and the index of neutrophilic maturation were examined [12, 24]. C57BL/6 mice were used in these studies following standard rules and regulations. Experimental animals (males C57BL/6 mice of 16-24 g) were divided into groups depending on the active ingredient used. The immunobiological activity of both drugs and their combination in various doses were determined. Induction of the endogenous interferon was analyzed in a model of staphylococcal infection in mice [25].

Statistical processing of the results was performed using the STATISTICA 6.0 application package (StatSoft, USA) using the Student's t-test and the Mann-Whitney U-test [7]. Significant differences between control and treatment groups were considered at $p < 0,05$.

In this study a three step chromatographically purified drug Laferobion (LLC «Pharmaceutical plant «Biofarma», Bila Tserkva, Ukraine) was used. The obtained renaturated IFN was first purified by using sorbent SM - Toyopearl-650M. On the

second stage of the chromatographic purification, the IFN solution was applied to the DEAE-Toyopearl-650M sorbent. The purification of the IFN monomer from the traces of the polymeric IFN forms were conducted by gel filtration on TSK-gel «Toyopearl» HW-55 resin. For comparison of the immunobiological effects, a highly purified by ion exchange chromatography and gel filtration staphylococcal adhesive protein (mW 70 kDa) was used.

The effects of the drug preparations on the production of interferons (IFN) in mice were evaluated by a micromethod, as described [8, 9].

Testing of the tumor necrosis factor (TNF-α) levels in animal serum was performed using a colorimetric cytotoxic test in L_{929} tumor line of transformed murine fibroblasts [10, 11].

Levels of cytokines in cell culture supernatants of cells activated with adhesive protein p70 were determined by ELISA. Commercial kits for the determination of TNFα, IFN were obtained from R&D Systems (USA). ELISA studies were performed in accordance with protocols developed by the manufacturer.

Results.

In the control group of mice, after a single injection of G-CSF, an increase in leukocyte count was observed. In animals with experimental leukopenia, drug combinations increased absolute leukocyte counts. At the same time, if Filstim and EAP were used separately no statistically significant increase in leukocyte counts was observed. Single-dose subcutaneous injection of Filstim and EAP drugs increased the number of myelocarocytes, eosinophils, monocytes and immature neutrophils in the bone marrow. In control and experimental groups, after application of the studied drugs, an increase in the absolute content of immature forms of neutrophils was observed. Such changes have led to a decrease in the index of maturation of neutrophils (table 1).

Table 1. Absolute white blood cell counts (WBC) (10^9/L) in the peripheral blood of the C57BL/6 mice after the 21-day treatment with Filstim and *Staphylococcus aureus* (EAP) cell wall biopolymer (M ± m).

	Eosinophils	Monocytes	Lymphocytes	Segmented Neutrophils	Band Neutrophils
Untreated Day 0	0,281±0,035	0,198±0,027	5,16±0,28	2,33±0,20	0,089±0,017
Untreated Day 21	0,285±0,039	0,203±0,036	5,12±0,32	2,37±0,23	0,082±0,013
Filstim (1 mg/kg)	0,315±0,047	0,339±0,041*	0,158±0,033	4,40±0,82*	0,158±0,033*
Filstim+EAP (1 mg/kg)	0,427±0,062*	0,487±0,082*	7,62±0,49*	5,73±0,85*	0,264±0,058*

Note: * $p < 0,05$ as compared to untreated animals.

The table shows that the absolute counts of the segmented and banded neutrophils were significantly increased in animals treated with Filstim + EAP at a dose of 1 mg/ kg as compared to Filstim alone. Similarly, the increase in monocyte counts were observed in Filstim+EAP group. An increase in lymphocyte counts was observed in animals trated with Filstim+EAP. Statistically significant increase in eosynophils was found after treatment with Filstim + EAP.

Also, the immunobiological activity of the drug Laferobion and highly purified and biochemically characterized staphylococcal biopolymer were examined. The experimental model for the examination of the endogenous interferon production was established in C57BL/6 mice. In animals injected with the physiological solution prior to staphylococcal infection, interferonogenesis was inhibited at day 3 post infection. The preventive use of EAP at 100 μg/mouse was the most effective and long-lasting, stimulated the production of IFN in infected animals. In addition to the increase in IFN, stimulation of TNF production was observed in serum of animals that were preventively injected with studied surface protein-antigen substances of staphylococci.

The wide spectrum of biological activity of the interferons causes is important for preservation of homeostasis in the organism, and our data provide evidence forboth direct and feedback relationship links between the systems of interferon, immune and neuroendocrine systems that interact in order to protect the organism. It is also important to note that due to the spread of viral pathogens, interferon-related drugs are in increasing demand.

To enhance the interferonogenesis and amplify effects of interferons, the coapplication of natural biopolymer EAP provides protective effect as an immunobiological drug. Our studies have demonstrated its effectiveness and safety.

Induced interferons support immune responses against a wide range of viral infections and involve cells that that express interferon receptors. Interferons induce both local and systemic antiviral responses in other cells as well. In addition, interferons have two other important properties: inhibition cell proliferation (and thus potentially an antitumor agent) and immunomodulatory effects.

Interferon inducers include various substances, such as the antiviral drug Herpevir (Acyclovir). The most important attribute of IFN inducers is their universal range of antiviral activity. The production of protective cytokines, including interferons, is also influenced by bacterial factors. Our studies were conducted using antigenic substances of ubiquitous staphylococci, such as the Staphylococcus aureus Wood 46 cell wall surface protein (the Czech Republic collection of microorganisms) in combination with the drug Herpevir. Using this experimental model, we identified protective effects of the investigated drugs on the production of endogenous interferon in C57BL/6 mice. In assessing the staphylococcal infection in control mice (injected with physiological solution 24 hours before the infection), we observed inhibition of interferonogenesis beginning from 3-4 days of the disease. For prophylactic applications, we evaluated the effects of individual drugs and their combined effect in the presence of Staphylococcus (EAP) in a dose of 100 μg/mouse. The most intensive and long-lasting stimulated IFN production in staphylococcus-infected animals was found with EAP. In addition to IFN production, we also observed increased TNF

production in the serum. These findings support our recommendations for immunoprophylactic use of these staphylococcal surface antigen substances.

Conclusions.

1. The combined effect of Filstim and EAP biopolymer complex has a pronounced hemostimulating effect in both intact mice and in animals with experimental leukopenia. The effect of the drug significantly increases the number of leukocytes in peripheral blood, as well as increases the number of myelocarocytes in the bone marrow.

2. When combined with staphylococcal biopolymer and G-CSF (Filstim), synergistic effects were observed on immunomodulatory properties, therefore, after studying toxicity, the resulting biopolymer can be recommended for clinical use.

3. In the evaluation of immunobiological activity of the combined preparation Laferobion and staphylococcal biopolymer in the experimental model of exposure to endogenous interferon products in the C57BL/6 mice, a protective effect was established.

4. In staphylococcal infection, the inhibition of interferonogenesis was observed in experimental mice, and the prophylactic use of EAP (100 μg/mouse) had the most intensive and long-lasting stimulatory effect on IFN production in the infected animals. Along with increased IFN production, stimulation of the TNF production in the serum was observed as well.

5. Our results demonstrate synergistic effects of combined drug application with the Staphylococcus biopolymer. The effect is targeted to the monocyte-macrophage system whose primary function is the elimination of microorganisms. The combination drug enhances the functional activity of these cells, stimulates phagocytosis and antimicrobial activity, activates the cytotoxic function of macrophages, which is manifested in their ability to destroy in vitro syngeneic and allogenic tumor cells. Activated monocytes and macrophages begin to synthesize some of the cytokines – IL-1, IL-3, TNF, colony-stimulating factors, IFN and others factors. These effects lead to the activation of humoral and cellular immune responses.

6. The proposed microbial biopolymer can be used in combinations with other biopharmaceutical agents with antiviral, immunostimulating and immunoregulatory effects in order to maximize the level of protective immunity.

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Стаття надійшла до редакції в листопаді 2018 року.