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Immunotropic Activity of a New Cladribine/Ribavirin-Based Composition

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Abstract

The immunotropic potential of the cladribine and ribavirin combination and its individual components has been studied applying the model of the cell-mediated immune response in F_1 (C57Bl/6 × CBA) hybrid mice, as well as the protective potential of the oral drug Leucovir (Leucovir) based on this combination using a model of Experimental Autoimmune Encephalomyelitis (EAE) in guinea pigs.

It has been demonstrated that the combination of cladribine and ribavirin has an immunosuppressive effect on the cell-mediated immune response, which manifests itself as inhibition of antigen-specific T-cells clone formation and inhibition of pro-inflammatory cytokines production. The combination of cladribine with ribavirin is superior in effectiveness to its individual components. The use of Leucovir makes it possible to successfully control the course of EAE, which reproduces Multiple Sclerosis (MS), both in preventive and therapeutic use. The preventive use of Leucovir significantly reduces the percentage of disease incidence and the mortality rate, allows delaying the onset of the first EAE signs, and reduces the clinical manifestations of the modeled pathology. The therapeutic use of Leucovir inhibits the progression of neurological impairments, reduces the mortality rate and contributes to a significant increase in the survival time after immunization.

These results suggest that Leucovir, a cladribine- and ribavirin-based oral drug, may have beneficial effects in autoimmune diseases such as MS.

Keywords: Cladribine; Ribavirin; Leucovir; Multiple sclerosis; Immunomodulation

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Introduction

Multiple sclerosis is a chronic inflammatory demyelinating disease of the Central Nervous System (CNS) that leads to progressive neurodegeneration and severe disability [1]. The main pathological manifestation of MS is sclerotic plaques displaying demyelination of the white and gray matter in the brain and spinal cord [2]. In such focal lesions, the destruction of myelin sheaths and oligodendrocytes occurs after they are recognized by immune system cells [3]. This inflammatory response is associated with the activation of myelin-specific CD4+ autoreactive cells and their differentiation into pro-inflammatory T-helper (Th) cells, as well as the activation of CD8+ T-cells and B-cells that secrete antibodies, cytokines and other immune system factors [1,4-6]. Thus, the hypothesis of the immune-mediated pathogenesis of MS is currently generally accepted.

MS arises from complex interactions between autoimmune processes, individual genetic susceptibility, and environmental factors such as viruses [7]. There is growing evidence of the possible role of viral infections (herpes simplex virus type 6, herpes zoster, Epstein-Barr virus, herpes simplex virus type 1, influenza virus, etc.) as a trigger of the autoimmune process in MS in a genetically susceptible population and/or disease relapse [7-9]. In MS, the isolation of viral particles from active plaques within the CNS, as well as the increased humoral and cellular immune responses to these viruses in the peripheral blood are strong arguments in the support of this hypothesis [8]. The results of numerous epidemiological studies clearly indicate that the viral reactivation is an important risk factor for MS and relapse [7]. Moreover, clinical trials data demonstrate a tendency towards a decrease in the number of new active lesions in MS patients with high Magnetic Resonance Imaging (MRI) activity treated with valacyclovir [8,10].

Considering the main role of autoimmune processes in the development of MS, current therapeutic approaches are associated with the use of immunosuppressive (IFN-β, glatiramer acetate) and immunomodulatory agents (mitoxantrone, cyclophosphamide, teriflunomide) [11,12]. In recent years, cladribine has been added to the arsenal of drugs with immunotropic action for the treatment of MS [13,14]. The possibility of using cladribine in this pathology is explained by

a combination of selective immunosuppressive action against mononuclear cells (lymphocytes, monocytes) and relatively low toxicity in comparison with other immunosuppressants [14].

Cladribine causes the selective depletion of T and B cells with a greater effect on CD4+ and CD8+ cells and has comparably low activity against other hematologic and immune cell types in MS patients that lead to an approximately 4-fold decrease in the CD4 +:CD8+ ratio [14- 16]. In term of lymphocyte depletion dynamics, the treatment with cladribine causes a sustained dose-dependent decrease of lymphocyte counts, followed by a gradual recovery [17,18]. In addition, cladribine also reduces of pro-inflammatory cytokines and chemokines levels in serum and cerebrospinal fluid [16].The use of cladribine in MS is accompanied by clinically relevant effects in term of a relapse rate reduction, as well as MRI lesions, oligoclonal bands expression in cerebrospinal fluid, and delay in disability progression in some cases [15,18-21]. At the same time, Cladribine therapy is associated with a relatively high rate of herpes infections and herpes zoster [12,22].

Ribavirin is a broad-spectrum antiviral agent, the activity of which has been demonstrated against influenza A and B viruses, herpervirus infections, cytomegalovirus, etc. [23]. The action of ribavirin entails both direct activity against viruses and modulation of the host immune response [24]. The immunosuppressive effect of ribavirin is also known, manifested by inhibition of the proliferation of T and B lymphocytes [24]. The protective properties of ribavirin have been shown in experimental models of autoimmune diseases, including EAE [24] and lupus nephritis [25]. Attenuation of nitrosative stress through the modulation of inducible Nitric Oxide Synthase (iNOS) can explain the neuroprotective properties of ribavirin since the iNOS overexpression is associated with neurodegenerative diseases, including MS [26].

In addition, ribavirin is an inhibitor of Inosine 5'-Monophosphate Dehydrogenase (IMPDH), one of the key enzymes of guanine nucleotides de novo biosynthesis [27]. The inhibition of IMPDH reduces the guanine nucleotide pool and makes it possible to suppress T- and B-cells proliferation in several types of autoimmune diseases [27]. Therapeutic effects of IMPDH inhibitors have been demonstrated in a broad range of autoimmune diseases, including rheumatoid arthritis, psoriasis, systemic lupus erythematosus, and MS [28].

Thus, despite the fact that ribavirin has been developed as an antiviral drug, the spectrum of its activity as an inhibitor of IMPDH and immunomodulatory effects indicates the therapeutic potential of ribavirin against autoimmune diseases.

The autoimmune etiology of MS and the role of viral infections in its development suggest that the combination of cladribine, which exhibits a selective immunosuppressive effect, and ribavirin, with a broad spectrum of antiviral activity, immunomodulatory and neuroprotective effects, may be an attractive therapeutical tool for the treatment of MS.

Many *in vitro* and *in vivo* test systems are used to confirm the immunotropic activity of drugs. The Delayed-Type Hypersensitivity (DTH) reaction is a manifestation of the cellular component of the immune response and is used to study primary inflammatory processes. After obtaining evidence of the immunotropic activity of the drug, it is important to assess its neuroprotective ability on the relevant model of MS - EAE.

In this study, the immunotropic effect of a cladribine and ribavirin combination and its individual components has been evaluated in a cell-mediated immune response model (DTH), as well as the protective potential of an oral drug Leucovir based on this combination using an animal model of MS (EAE).

Materials and Methods

Animals

The study of the immunotropic activity of cladribine and ribavirin, their combination has been carried out on F_1 (C57Bl/6 × CBA) female hybrid mice weighing 18 g to 20 g, aged 2 months to 3 months (Institute of Bioorganic Chemistry of the National Academy of Sciences of Belarus, Republic of Belarus). EAE modeling has been carried on male guinea pigs weighing 250 g to 350 g (Republican Scientific Research Unitary Enterprise "Institute of Experimental Veterinary Medicine named after S.N. Vyshelessky" of the National Academy of Sciences of Belarus, Republic of Belarus). All experiments have been performed in compliance with international ethical guidelines and national requirements of using laboratory animals.

Treatment

Cladribine and ribavirin, as well as their combinations with different ratios of components (ChemPharmSintez SPC, Institute of Bioorganic Chemistry of the National Academy of Sciences of Belarus, Republic of Belarus) have been used in an experimental model of the cell-mediated immune response. Cladribine was administered to animals once at doses of 1 mg/kg, 2 mg/kg, 4 mg/kg and 8 mg/kg, while ribavirin was administered once at doses of 100 mg/kg, 200 mg/kg, 400 mg/kg and 800 mg/kg. The combination of Cladribine and ribavirin was prepared in a ratio of 1:100 and was administered once at doses of 51 mg/kg, 101 mg/kg, 201 mg/kg, and 402 mg/kg in terms of the total amount of active ingredients.

Leucovir, enteric-coated tablets containing 1 mg of cladribine and 100 mg of ribavirin (ChemPharmSintez SPC, Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, Republic of Belarus), was used in the study on the EAE model. The animals were injected with a crushed tablet mass at a dose that demonstrated the highest activity in a model of a cellular immune response. In this case, an equivalent dose was used taking into account the interspecies dose transfer (70.5 mg/kg) [29]. Leucovir was administered for 7 consecutive days.

All studied samples were administered intragastrically (ig) to experimental animals using a special probe.

Model of delayed-type hypersensitivity

The effect of the studied samples on the cell-mediated immune response has been studied using the DTH model. This model was used to evaluate the effects of cladribine (1 mg/kg, 2 mg/kg, 4 mg/kg and 8 mg/kg), ribavirin (100 mg/kg, 200 mg/kg, 400 mg/kg, and 600 mg/kg) and their combinations (51 mg/kg, 101 mg/kg, 201 mg/kg, and 402 mg/kg). In the experiments, F_1 (C57Bl/6 × CBA) mice have been used. Each group included 6 animals.

DTH reaction was induced using an antigen - freshly isolated Sheep Red Blood Cells (SRBC) [30]. For sensitization, SRBC (2×10^5) were injected into the tail vein of mice of all groups on Day 0. The test samples were administered simultaneously with a sensitizing and/or challenge dose of antigen. On Day 4 after sensitization, all mice were

challenged with antigen (1× 10^8) under the aponeurotic plate of one of the hind paws. A sterile 0.9% sodium chloride solution was injected into the contralateral paw in the same volume (control). Animals of the control group received placebo instead of the test drugs. 24 h after the SRBC challenge (Day 5); the thickness of the SRBC rechallenged paws and Normal Saline (NS) rechallenged paws were measured (with a micrometer). Then the intensity of the inflammatory reaction was determined by calculating the percent swelling of the paw by formula [31]:

$$\text{Percent swelling of the paw} = \frac{\text{Thickness (SRBC rechallenged paw)} - \text{Thickness (NS rechallenged paw)}}{\text{Thickness (NS rechallenged paw)}} \times 100\%$$

Experimental autoimmune encephalomyelitis

EAE is the most relevant model of MS and is widely used as a method for studying the pathogenesis of the disease and evaluation of potential therapies for MS [32]. EAE has clinical and pathogenetic manifestations similar to MS: Inflammation, demyelination, axonal loss, and gliosis [33]. This model is an autoimmune disease characterized by the inflammation-associated infiltration of the CNS by T cells and monocytes [32]. Molecular targets are proteins expressed by myelin-producing oligodendrocytes in the CNS [32]. The result is primary demyelination of the axonal tracks, impaired axonal conduction in the CNS, and progressive hind limb paralysis [32].

The EAE model was used to study the protective potential of Leucovir - the cladribine and ribavirin combination-based drug product. In experiments, guinea pigs have been used, which are considered one of the animal models that best reproduce MS [33].

Modeling of the pathological state was performed by the intradermal inoculation of encephalolytic mixture to experimental animals. An encephalolytic mixture containing 100 mg of homologous spinal cord tissue homogenate, 0.1 ml of 0.9% sodium chloride solution and 0.15 ml of Freund's complete adjuvant (Sigma-Aldrich) with Mycobacterium tuberculosis (5 mg/ml) was injected once into the right hindpaw at a total volume of 5 μg.

Guinea pigs sensitized with encephalolytic mixture were randomly assigned into 3 groups (n=15 in each group). The animals of the control group were injected with placebo, the rest received Leucovir at a dose of 70.5 mg/kg in two treatment protocols: From the start of immunization (preventive treatment) or from the onset of the first EAE signs after the induction (therapeutic treatment).

Disease latent period between immunization and EAE's symptom onset, the percentage of disease incidence, the mortality rate, as well as the survival time after immunization was assessed. To assess the severity of EAE 13, 18, 22, 27, 30 and 35 days after immunization, clinical signs of EAE were scored as follows: No changes (0), weak changes (1), mild EAE (2), moderate changes (3), severe changes (4), and severe changes with a lethal outcome (5). The mean clinical score for each group was calculated.

Statistical analysis

The results are presented as mean ± Standard Error (SE). Statistically significant differences between experimental groups were assessed using the Student's t-test for the data that follow a normal distribution. Chi-square and Fisher's exact tests were used to assess the percentage of disease incidence and lethality. The level of statistical significance was P<0.05. Statistical analysis was performed using Microsoft Excel and Statistics.

Results and Discussion

The effect of cladribine, ribavirin and their combination on the cell-mediated immune response in the model of delayed-type hypersensitivity

The antigen administration to sensitized animals of control groups was accompanied with pronounced paw swelling (31.7% to 43.8%), which indicated the induction of DTH response associated with the reactivity of type 1 T-helper cells (Th1) [34] (Figure 1A). The use of cladribine in the dose range of 1 mg/kg to 4 mg/kg simultaneously with a sensitizing dose of antigen was accompanied by significant suppression of the DTH reaction (Figure 1A). When using Cladribine at a dose of 8 mg/kg, the inflammatory response was comparable to that in the control. This beneficial effect of cladribine was obviously more profound at doses of 2 mg/kg and 4 mg/kg (Figure 1A).

Ribavirin in the dose of 200 mg/kg to 600 mg/kg, administered simultaneously with sensitization of animals, also demonstrated a significant suppressive effect on DTH reaction (Figure 1B). The effect was most pronounced when using a dose of 400 mg/kg, whereas the lowest dose (100 mg/kg) was ineffective.

The results obtained indicate the suppressive effect of cladribine (1 mg/kg to 4 mg/kg) and ribavirin (200 mg/kg to 600 mg/kg) for the formation of an antigen-specific T-cell clone during a cell-mediated immune response.

Further, this model was used to study the effect of cladribine and ribavirin combination (ratio 1:100) on the cellular immune response. Compositions No. 1-4 at doses of 51 mg/kg, 101 mg/kg, 201 mg/kg and 402 mg/kg, respectively, administered simultaneously with

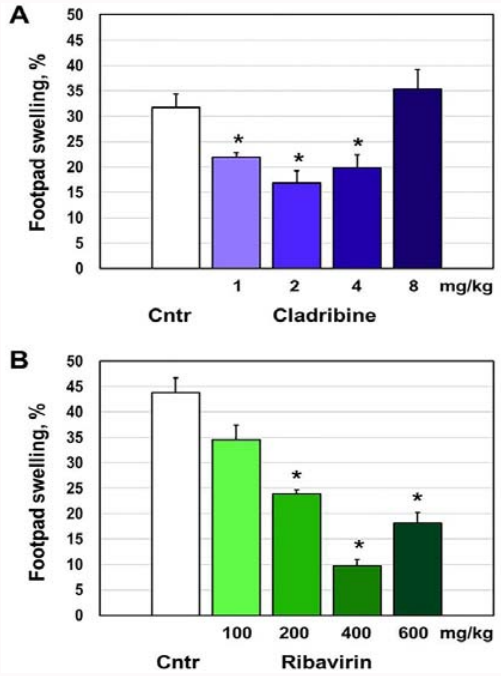

Figure 1: Effect of cladribine and ribavirin in the development of Delayed-Type Hypersensitivity (DTH) in mice F_1 (CBA × C57Bl/6). **A:** Effect of cladribine on the development of DTH in mice F_1 (CBA × C57Bl/6). **B:** Effect of ribavirin on the development of DTH in mice F_1 (CBA × C57Bl/6). Data are represented as mean ± Standard Error (SE). Cntr - Control. *P<0.05 *vs.* control, n=6.

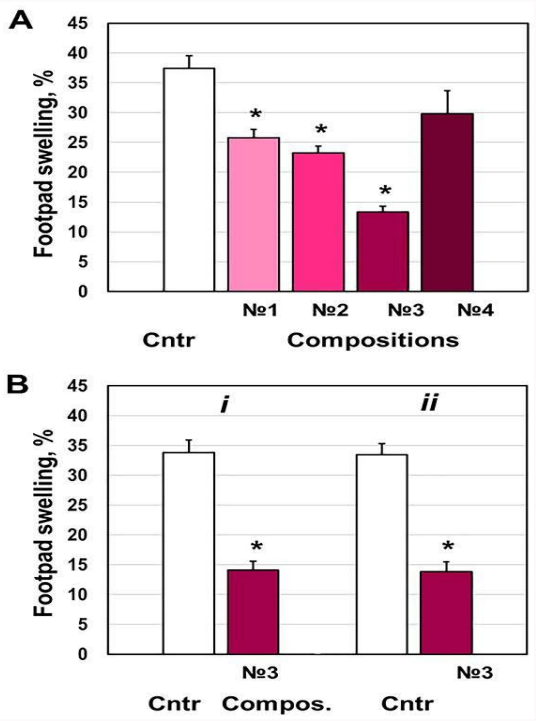

Figure 2: Effect of different combination of cladribine and ribavirin in the development of Delayed-Type Hypersensitivity (DTH) in mice F_1 (CBA × C57Bl/6). **A:** Effect of different combination of cladribine and ribavirin (composition №1-4) on the development of DTH in mice F_1 (CBA × C57Bl/6). Combination of cladribine and ribavirin (1:100): composition №1 (51 mg/kg), №2 (101 mg/kg), №3 (201 mg/kg), and №4 (402 mg/kg). **B:** Effect of composition №3 on the inductive (*i*) and effector (*ii*) phases of DTH response in mice F_1 (CBA × C57Bl/6). Data are represented as mean ± Standard Error (SE). Cntr - Control, Compos - Composition. *P<0.05 *vs.* control, n=6.

sensitization, were evaluated. Inhibition of DTH was demonstrated with the use of compositions No. 1-3, which was expressed by a significant decrease in paw swelling (Figure 2A). The greatest suppressive activity was registered with the administration of composition No. 3 (201 mg/kg). A further increase in the dose to 402 mg/kg (composition No. 4) led to a sharp drop in the combination activity.

The results of the study suggest that the combination examined at a dose of cladribine not exceeding 1 mg/kg has a dose-dependent immunosuppressive effect in the DTH model. Taking into account the data obtained when assessing the effect of individual components of the combination on a similar experimental model, one may talk of an additive effect for the combination usage. The rate of suppression of the inflammatory response in this model with the administration of a cladribine and ribavirin combination is more than 2 times higher than its value in the case of using cladribine and ribavirin separately.

For further study, the composition No. 3 (cladribine + ribavirin, 201 mg/kg) was selected, which demonstrated the highest activity in screening. Its effect on various stages of the cellular immune response of DTH was evaluated: On the clone formation of antigen-specific T-lymphocytes, as well as on the ability of these lymphocytes to produce pro-inflammatory cytokines when meeting an antigen.

For this purpose, some of the animals received the selected combination (cladribine + ribavirin, 201 mg/kg) simultaneously with a sensitizing dose of antigen (day 0), the rest of the mice were administered this combination after injection of a challenge dose of antigen (Day 4).

The experimental data show the ability of the combination studied to influence all the assessed stages of the cellular immune response (Figure 2B). Composition No. 3 markedly reduced the intensity of the inflammatory reaction both when it was used simultaneously with the administration of an inducing dose of antigen (Figure 2B, i), and in the case of its administration simultaneously with a challenge dose of antigen (Figure 2B, ii). These results indicate that cladribine and ribavirin combination (201 mg/kg) inhibits the Th1-mediated cellular immune response, including the formation of a clone of antigen-specific T-lymphocytes and the production of pro-inflammatory cytokines, which play a leading role in the MS pathogenesis [35].

The demonstrated ability of cladribine and ribavirin combination not only to suppress the Th1-cells differentiation, but also to inhibit the production of pro-inflammatory cytokines seems to be a very important aspect of its therapeutic potential. The fact is that inhibition of the production of cytokines involved in demyelination processes in focal inflammatory lesions allows shifting the balance in favor of anti-inflammatory cytokine profile, which is one of the approaches used in MS therapy (INF-β, daclizumab) [35].

Hermann et al. [36] notes that compounds that require intracellular phosphorylation to become active, such as lamivudine, zalcitabine, ribavirin, stavudine, and zidovudine, should not be administered concomitantly with cladribine in the treatment of MS. This statement is based on a case report on the interaction of cladribine and lamivudine in a patient with B-cell chronic lymphocytic leukemia, as well as the results of *in vitro* studies demonstrating the ability of lamivudine to inhibit intracellular phosphorylation of cladribine, which may be associated with a risk of reducing its effectiveness in the treatment of lymphoproliferative diseases [37].

At the same time, there is an assumption that phosphorylation of cladribine by deoxycytidine Kinase (dCK) is not the only mechanism explaining the activity of cladribine [38,39]. Prevention of cladribine phosphorylation with an excess of deoxycytidine does not alter the inhibition of cytokine secretion by lymphocytes, observed upon exposure to cladribin [38]. These effects can be explained by the ability of cladribin, as an adenosine derivative, to bind to adenosine receptors [39,40], which are implicated in modulating the immune response [41].

The results of our study showed that the use of cladribine with ribavirin, and, possibly, with other nucleosides having an antiviral effect, for the activation of which phosphorylation by dCK is required, demonstrates an additive effect with the increase in the suppressor effect by more than 2 times in comparison with the total effect of individual components.

Experimental allergic encephalomyelitis

The clinical manifestation of EAE in animals of the control group was characterized by the early onset of the first symptoms on average on the 15th day (Table 1). EAE symptoms were typical for all control animals and progressed from mild to severe, which led to the death of most (13/15) vehicle-treated animals (Table 1). At the same time, in the control, general weakness, impaired coordination of movements, paresis and paralysis of the sphincters and hind limbs, dysfunction of the pelvic organs (urinary and fecal incontinence) were reported. The mean survival time of control animals after immunization was 10.9 ± 1.0 days.

Table 1: Neuroprotective activity of Leucovir (70.5 mg/kg, 7 d) in the experimental autoimmune encephalomyelitis model in guinea pigs (mean ± SE).

Group	Disease's latent period, day	The percentage of disease incidence	The mortality rate	Survival time after immunization, days
Control	15.2 ± 0.8	15/15	13/15	10.9 ± 1.0
Leucovir, preventive treatment	20.0 ± 1.5*	6/15*	3/15*	21.0 ± 3.5*
Leucovir, therapeutic treatment	16.9 ± 1.5	14/15	8/15	18.4 ± 1.3*

*: P<0.05 *vs.* control

Table 2: Effect of Leucovir (70.5 mg/kg, 7 d) on clinical signs of the experimental autoimmune encephalomyelitis in guinea pigs.

Group	The mean clinical score, days post of EAE immunization					
	13 d	18 d	22 d	27 d	30 d	35 d
Control	0.1	1.3	3	4	4.2	4.3
Leucovir, preventive treatment	0	0.2	0.6	1.3	1.4	1.4
Leucovir, therapeutic treatment	0.3	0.9	1.3	2.3	2.4	2.5

The administration of Leucovir, starting from the day of immunization (preventive treatment), significantly changed the EAE manifestation in animals. Disease's latent period was significantly lower in comparison with the control (Table 1). EAE signs of varying grades developed only in 6/15 Leucovir-pretreated animals. A pronounced amelioration of the EAE clinical course was also noted, as evidenced by lower mean clinical score values than in control animals throughout the observation period (Table 2). In addition, the administration of Leucovir led to a significant decrease in the mortality rate and an increase in the survival time after immunization in comparison with the vehicle-treated EAE group (Table 1).

In the group of animals that received Leucovir since the onset of EAE symptoms (therapeutic treatment), the disease's latent period was comparable to the control (Table 1). EAE was successfully simulated in almost all animals in the group (14/15). At the same time, the administration of Leucovir contributed to a pronounced decrease in the disease manifestation, as evidenced by the lower mean clinical score values in comparison with the control, registered on days 18 to 35 after immunization (Table 2). For animals treated with Leucovir, the survival time after immunization markedly increased in comparison with the vehicle-treated animals, and a tendency towards a decrease in mortality rate was reported (Table 1).

The improvement in the general condition of experimental animals under the influence of Leucovir is evidenced by the data of body weight dynamics registration. In case of preventive administration of Leucovir, a positive dynamics of the body weight of the animals was reported; in the case of the therapeutic treatment, a less pronounced body weight loss was observed in comparison with the control.

Significant differences between the results obtained for Leucovir-treated, Leucovir-pretreated and control animals indicate the favorable effects of the preventive and therapeutic use of Leucovir in the EAE simulation in experimental animals and may indicate the neuroprotective properties of the studied drug.

Probably, the observed decrease in EAE manifestations is due to proven neuroprotective effect in one of the components of the combination (ribavirin). In a study by Milicevic et al. [24] (2003), the intraperitoneal (ip) administration of ribavirin led to a decrease in the EAE signs and a reduction in the manifestation of the disease, while the neuroprotective effects of ribavirin were most pronounced with the preventive use of ribavirin [24]. In addition to these data, histological studies showed that the use of ribavirin in the spinal cord tissues decreased the infiltrates of mononuclear cells, consisting of T-cells and macrophages, and there were no signs of demyelination, which were characteristic of control animals with EAE [24]. It should be noted that ip administration of cladribine to SJL/J mice was not effective against the clinical symptoms of EAE [42].

The main purpose of combination therapy is to provide a higher treatment efficacy compared to the use of individual drugs. This study is the first one to obtain data indicating a more pronounced suppressive effect of the cladribine and ribavirin combination on the cellular immune response than the individual components of the combination. The mechanism of the additive action of this combination is currently not exactly known and requires study. The combination of cladribine and ribavirin suppresses the Th1-mediated cellular immune response, including the formation of a clone of antigen-specific.

T-lymphocytes and the production of pro-inflammatory cytokines that play the important role in the pathogenesis of MS [35].

The ability of Leucovir, containing a combination of cladribine and ribavirin, to have a pronounced effect on the symptoms of EAE, in the pathogenesis of which the Th1-cells play a key role [6], also confirms the information obtained in DTH model about its action mode. The beneficial effects of the preventive and therapeutic use of Leucovir on the development of a simulated pathology reproducing the course of MS indicates that the combination of cladribine and ribavirin can be considered as a therapeutic option in the treatment of MS.

Conclusion

The study has established the immunosuppressive effect of the cladribine and ribavirin combination (51 mg/kg to 201 mg/kg, ig, single dose) on the cell-mediated immune response in DTH model in F_1 (CBA × C57Bl/6) hybrid mice. When using the combination at a dose of 201 mg/kg, the pharmacological effect of the combination was more pronounced than the action of its individual components (additive effect), and manifested itself both in the form of inhibition of the formation of a clone of antigen-specific T-lymphocytes and in the suppression of the pro-inflammatory cytokines production.

The drug product Leucovir (70.5 mg/kg, ig, 7 d) developed on the basis of a combination of cladribine and ribavirin makes it possible to successfully control the course of EAE in guinea pigs that reproduce MS in humans, both in preventive and therapeutic treatment.

The data obtained in the study are of important practical interest and allow considering the studied combination as a perspective

approach for further improvement in MS therapeutics.

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