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EXPERIMENTAL STUDIES

Antirheumatic Activity of Methotrexate in Phospholipid Nanoparticles (Phosphogliv)

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Abstract—The efficiency of methotrexate use in the basic therapy of rheumatoid arthritis is limited because of risk of side effects and fast drug efflux from zone of joints as well. We have developed a new stabilized form of methotrexate using phospholipid micelles of the injection form of the Phosphogliv preparation as a carrier. Phosphogliv has recently been developed in the Institute of Biomedical Chemistry (Moscow), as the emulsion of 50 nm phospholipid nanoparticles stabilized by glycyrrhizic acid. The conditions of maximal methotrexate incorporation into the phospholipid nanoparticles were optimized under control of HPLC (60% of total methotrexate was associated with nanoparticles). Methotrexate in phospholipid nanoparticles exhibited higher therapeutic efficiency in experimental adjuvant arthritis in rats than with free methotrexate. (This was evaluated by the decrease of edema and swelling of joints and inhibition of secondary inflammatory reaction.) The increase of antirheumatic activity of the developed preparation may also be attributed to the influence of glycyrrhizic acid, possessing both anti-inflammatory and immune properties. It is suggested to use a new form of methotrexate for intra-articular administration for rheumatoid arthritis treatment.

Key words: rheumatoid arthritis, methotrexate, nanoparticles, phospholipids, phosphatidylcholine, glycyrrhizic acid.

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INTRODUCTION

Recent progress in the development of new pharmacological preparations is determined not only by design of new drugs but also by (with) modification of medicinal forms of known pharmacological substances for maximal improvement of such their properties as the increase of bioavailability and the decrease of side effects. Such modifications attract much attention due to intensively developing nanotechnologies [1], particularly, incorporation of a medicinal compound into nanoparticles. The resultant new forms of known medicinal preparations exhibit the same pharmacological effects and minimal (if any) side effects typical for free drugs (see reviews [1–3]). This is especially interesting in the case of cytostatic drugs possessing severe side effects. Methotrexate (MT) is one of them.

Methotrexate suppresses DNA synthesis by inhibiting dihydrofolate reductase, the enzyme involved into biosynthesis of pyrimidine nucleotides [4]. This underlines its antitumor activity, which is also attributed to increased expression of tumor cell folate receptors exhibiting affinity to MT [5].

Studies on preparation of various liposomal forms of MT are carried out for more than 10 years to improve pharmacokinetic properties of MT, including the increase of its efficiency and the decrease of side effects. Some researchers employ laboratory methods for chemical construction of "pro-drug" based on synthesized lipophilic MT derivatives with diglyceride [6] or a phospholipid [7], incorporated into liposomes. Gao and Jiang [8] synthesized polybutylcyanoacrylate nanoparticles of 100 nm loaded with MT for treatment of central nervous system tumors. Others synthesized paramagnetic nanoparticles of iron oxide covalently bound to MT via aminopropyl trimethoxysilane; using HeLa cells it was demonstrated that these nanoparticles released MT in lysosomes at low pH [9]. Kukowska-Latallo et al. used dendrimer nanoparticles conjugated with folic acid as the MT carrier; this increased affinity of MT to tumor folate receptors. Administration of such nanoparticles to rats with epithelial cancer caused their high accumulation in the tumor cells and increased therapeutic activity of MT accompanied by marked decrease of toxicity compared with free MT [5]. There are also reports on employment of conjugates with dextran-peptide [10], solid lipid nanoparticles based on stearic acid as the nanoparticles containing MT [11]. The use of nanoparticles increased MT circulation time.

Since the beginning of nineties MT (the peroral or more frequently the injection form) is also used for basic therapy of rheumatoid arthritis [4, 12]. Employment of MT for treatment of patients with inflammatory diseases of joints is determined by the fact that connec-

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tive tissue (preferentially joints) damage in rheumatoid arthritis results from immunopathological reactions (auto-aggression), which share some similarity with tumor diseases [13]. The antiarthritis and antirheumatic effects of MT are also determined by suppression of NO production by peritoneal macrophages [13], activation of production of anti-inflammatory cytokines (interleukins 8 and 14) [14] and also by the effect on altered metabolism of bone tissue [15].

However, in spite of these positive effects MT has limited applicability because of high hepatotoxicity of the peroral and injection forms of this drug [16]. Local intra-articular administration of MT also does not give requested results due to short time presence of MT in the articular cavity i.e., accelerated resorption.

Using dexamethasone and hydrocortisone some authors demonstrated that the rate of drug resorption from the articular cavity was lower in the case of employment of their liposomal forms. Later it was found [19, 20], that liposomal MT intravenously and intramuscularly administered to rats or intra-articularly administered to rabbits with experimental arthritis was more effective than the free drug in inhibiting synovial hyperplasia and cell infiltration. The increased anti-inflammatory effect was also observed in the case of liposomes containing a phospholipid conjugate with MT [21].

However, all these studies have not culminated yet in the development of real new optimized medicinal form of MT suitable for intra-articular administration and ready for practical use.

The preparation Phosphogliv has been developed in Institute of Biomedical Chemistry, Russian Academy of Medical Sciences and introduced into clinical practice for treatment of liver diseases. The injection form of this drug represents nanoparticles (30–50 nm) stabilized by glycyrrhizic acid [22, 23]. Characteristics of this phospholipid nanosystem are potentially interesting for employment of Phosphogliv for optimization of pharmacological properties of other drugs, particularly, MT.

Possibility of MT incorporation into phospholipid particles is determined by the presence of its two carboxyl groups, which can interact with choline head group of phosphatidylcholine molecule in a weakly alkaline medium. The other Phospholiv component, glycyrrhizic acid, exhibits anti-inflammatory, antiallergic, and immunomodulating properties; it also acts as a synergist of glucocorticosteroids, which may therefore increase actirheumatic activity of MT [23].

Thus, the goal of this study was to investigate efficiency of MT incorporated into the phospholipid nanoparticles of the injection form of Phosphogliv using experimental adjuvant arthritis in rats and to compare its efficiency with that of free MT.

METHODS

Conditions for maximal MT inclusion into phospholipid particles have been recently developed [24]. Methotrexate (18 mg) dissolved in 0.01 M $NaHCO_3$ was mixed with initial substances used for Phosphogliv preparation: 0.5 g soybean phosphatidylcholine, Lipoid S100 (Lipoid, Germany), 0.2 g glycyrrhizic acid trisodium salt (Zhangjiagang Free Trade Zone Mafco Liantai Biotech Co., Ltd, China), and 2 g maltose monohydrate. The resultant aqueous mixture was homogenized for 5 min using a high pressure RANNIE MiniLab 7.30VH homogenizer (Denmark) For evaluation of the degree of MT incorporation into phospholipid particles, these particles were separated by gel filtration onto Sephadex G-25 and eluted with 0.2% ammonium acetate. The material eluted as a peak with a retention time 7.07 min was collected. After its lyophilization and phospholipids removal, the residue was dissolved in 10% aqueous solution of acetonitrile, and MT content was analyzed by HPLC using a Millichrom A-02 HPLC system and a gradient of acetonitrile from10 to 90%. Concentration of MT was evaluated by UV absorption at 258 nm using a calibration curve. Using this approach the amount of MT included into phospholipid particles represented 60% of total MT in the preparation. After homogenization the preparation was filtered through the filter of 0.45 μm, dispensed into vials (10 ml per vial) and lyophilized using the laboratory lyophilizer LyoLab F.

The size of phospholipid vesicles evaluated by means of a laser correlation spectrometer Intoks (Russia) was about 50 nm; the light transmission T at 660 nm (allowing evaluation of homogeneity of the preparation) was up to 75%. Both parameters remained the same after lyophilization and repeated dissolution.

For evaluation of biological efficiency the vial content was dissolved into 10 ml of water for injections just before use. The biological efficiency of free MT and MT included into the Phosphogliv nanoparticles was evaluated using the model of adjuvant arthritis in rats with marked inflammatory process accompanied by significant dystrophic changes in the articular cartilage.

Adjuvant arthritis in rats was modeled using the vaccine mycobacterium strain BCG-1. Suspension of mycobacteria in mineral oil was injected subplantar into the right hind paw. The primary inflammatory and secondary immunologic reactions were evaluated during 30 days by changes in an articular volume of injected and non-injected hinds. The oncometric evaluation of the articular volume employed a pletismograph (Ugo Basile).

Methotrexate was injected into the ankle-joint of anesthetized animals once a week in the dose of 0.18 mg. A single dose of the tested preparations (0.1 ml) contained the same amount of MT and also corresponding amount of Phosphogliv (5.0 mg phosphatidylcholine and 2.0 mg glycyrrhizic acid trisodium salt). Animals were treated for 3 weeks starting from

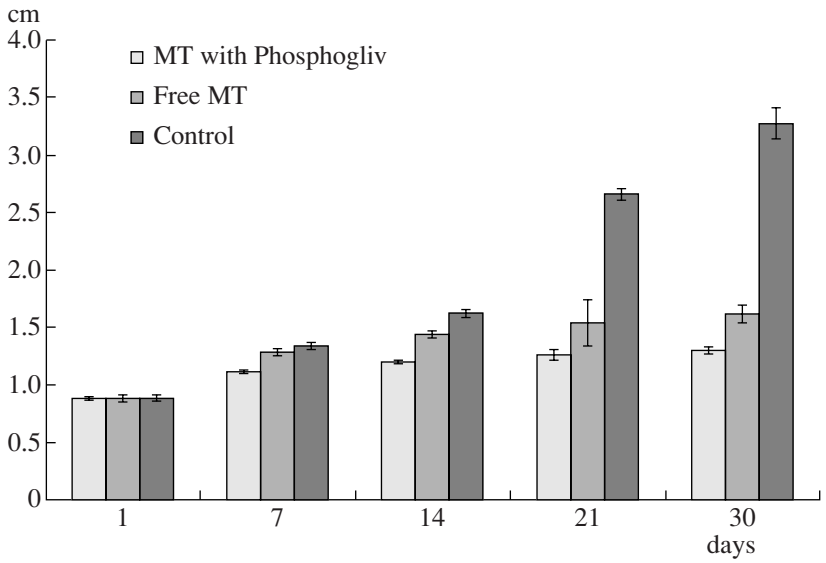

Fig. 1. The effect of administration of free methotrexate and methotrexate with Phosphogliv on time course of inflammatory reaction (the increase of swelling) in the injected paw in rats with experimental adjuvant arthritis.

the 14–15th day after BCG administration. (This period was characterized by fully formed signs of polyarthritis in experimental animals.) Rats were subdivided into 3 groups (9–10 animals per group): (1) rats received free MT; (2) rats received MT incorporated into Phosphogliv; (3) control (animals did not receive any medical treatment of polyarthritis). Inflammatory reaction and efficiency of therapy was evaluated by the increase of ankle joint volumes, changes in body mass etc. In the end of these experiments we investigated morphology of ankle joints.

RESULTS AND DISCUSSION

Although both forms of MT decreased inflammatory reaction in the injected hind, however, MT incorporated into Phosphogliv (i.e., the phospholipid form of MT) was more effective (Fig. 1). Administration of free MT during the period of experiment caused gradual (but lower than in control) increase of inflammatory reaction, whereas in animals treated with MT included into Phosphogliv the increase of inflammatory reaction stopped after the 14th day of the experiment and the size of the injected hind remained unchanged.

Methotrexate included into the phospholipids nanoparticles exhibited higher activity in inhibition of secondary immunologic reaction evaluated by classical signs of inflammation: hind edema, hyperemia and pain (Fig. 2).

Effect of free MT appeared on the 7th–8th and on the 10th day of treatment such therapy resulted in 40–50% inhibition of inflammation compared with control. In the case of MT included into Phosphogliv inhibitory effect appeared in the very beginning of treatment and time interval required for 40–50% inhibition of inflammation was two times shorter compared with the effect of free drug. The treatment with MT included into Phosphogliv for 10 days caused inhibition of the inflammatory reaction by 64%.

Thus, weekly intra-articular injections of MT included into phospholipid nanoparticles caused significant inhibition of both acute and secondary inflammatory reactions of ankle joints in rats. The antiarthritic effect of such medicinal composition significantly exceeded the effect of free MT.

Positive effect of this preparation has also been detected during morphological evaluation of hind joints in experimental animals. For example, we found significant decrease of synovial hyperplasia. Synoviocytes

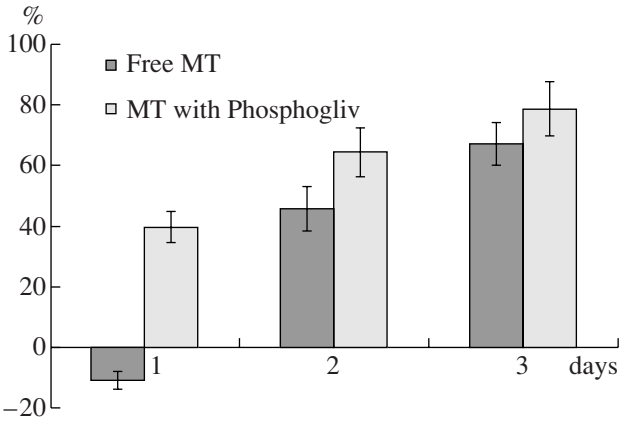

Fig. 2. Inhibition (%)of secondary immunologic reaction by free methotrexate and methotrexate with Phosphogliv in rats with experimental adjuvant arthritis. The decrease of edema in non-injected paw versus control at each time interval was defined as 100%.

formed only 1–3 layers (instead of 4–6 layers in control) without fibrin deposits and with weak manifestation of angiomatosis as well as weak infiltration with lymphocytes and histiocytes detected in other groups of animals. In hyaline cartilage from rats treated with MT included into Phosphogliv we found only small nidi of basophils of the interterritorial matrix (in contrast to marked fibrous cartilage and basophilia typical for the other groups of rats).

Data obtained indicate marked anti-inflammatory effect of this drug composition, reduction of the inflammatory reaction and proliferation of synoviocytes and blood vessels.

Thus, the results of this study suggest therapeutic efficiency of MT included into Phosphogliv; this may be attributed to the increase of MT exposure into articular cavity. Higher therapeutic potential of this composition may be also explained by the presence of glycyrrhizic acid, exhibiting anti-inflammatory and immunomodulating properties [23]. In addition, intra-articular administration of Phogphogliv increases the ratio phosphatidylcholine/ lysophosphatidylcholine in synovial fluid (because phosphatidylcholine is the main component of Phosphogliv). It should be noted that there is reverse interrelationship between this ratio and manifestation of rheumatoid arthritis symptoms [25].

Results of this study suggest that the developed form of MT included into Phosphogliv may significantly increase efficiency of treatment of patients with rheumatoid arthritis by local intra-articular therapy.

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