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Central action of some cytostatics – methotrexat (MTX) and doxorubicin (DXR). I. Long-term influence on the pain sensitivity and activity of brain dopaminergic system in mice

Ośrodkowe działanie niektórych cytostatyków – metotreksatu i doksorubicyny. I. Wpływ na wrażliwość bólową i aktywność układu dopaminergicznego w mózgu myszy

Neoplasms are still among the most frequent causes of human mortality, therefore the great interest of scientific laboratories around the world is focused on the searching for the new cytostatic drugs (7, 10, 11, 13). The antimetabolite of folic acid, methotrexate (MTX) and the antracycline antibiotic, doxorubicin (DXR) have long been major components of cancer treatment, as they both are highly effective chemotherapeutic agents, widely used in clinical medicine (3, 5, 9, 14, 16, 17). However, the evident efficacy of MTX and DXR is seriously limited by life-threatening myelosuppression and of DXR in addition by cumulative cardiotoxicity (3, 14, 15, 19). Both drugs administered in high single doses in the way of treatment of acute leukaemia developed their long-lasting toxicity during several days, manifested by the decrease of the body weight, myelosuppression, immunosuppression and the destruction of the epithelium of the gastrointestinal tract (3, 14, 16, 17). Both drugs are able to completely inhibit DNA synthesis and induce cell death in histocultures of tumors obtained via surgery (5). However, little is known about the central action of these substances.

The aim of the present studies was to investigate the influence of MTX and DXR on the central nervous system in the respect of their analgesic properties, inhibition of locomotion and exploratory activity and the ability to modulation of the brain dopaminergic system.

MATERIAL AND METHODS

The experiments were carried out on male and female Albino Swiss mice (20-25g). Standard food and water were available *ad libitum*. Each experimental group consisted of 12 animals. The central action of two cytostatics: methotrexat (MTX), antimetabolite of folic acid and doxorubicin (DXR), oncostatic antibiotic belonging to antracycline group, was studied. The investigated substances were dissolved in 0.9% NaCl and injected intraperitoneally (*ip*) in single doses 5 or 10 mg/kg in a constant volume of 5 ml/kg. The control animals received an equivalent volume of solvent. The behavioural experiments were performed 1 h, 7 and 14 days and biochemical analysis 5 h and 14 days after a single injection of drugs.

The substances, the solvents used for their dilution and way of administration are listed below: Doxorubicin (Adriablastine, Pharmacia, Italy) was diluted with saline and injected *ip*. D,L-amphetamine (Psychedrinum, Polfa) was suspended in 3% Tween 80 and injected *sc*. Glacial acetic acid (POCH, Gliwice) was diluted with distilled water to the concentration of 3% and administered *ip*. Haloperidol (Janssen) was injected *ip* as aqueous solution. Methotrexat (Rhone-Poluenc Rorer, France (was dissolved in saline and administered *ip*. Exact doses of the substances are given under the description of each test.

Animals were weighed at the begining of the experiment and at 7^{th} and 14^{th} day. Body weight was expressed in g as means of the group ± SEM.

Motor coordination was measured according to the method of G r o s s and T r i p o d (8). Mice were placed for 2 min on the rod rotating with the speed of 4 rpm. The amount of mice holding on the rod was noted. The effects were evaluated 1 h, 7 and 14 days after MTX or DXR administration.

Spontaneous locomotor activity of mice was measured in circular photoresistor actometers (32 cm in diameter). Animals were placed for 30 min in the actometers 1 h, 7 and 14 days after the drugs administration. Each crossing of the light beam was recorded automatically. Mean numbers of registered impulses ± SEM was considered as a spontaneous locomotor activity.

Exploratory activity in mice was investigated in a hole-board test according to the method of B o i s s i e r et al. (1). Animals were placed individually on the board (35x35 cm with 16 holes 28 mm in diameter) of a photocell actometer 1 h, 7 and 14 days after MTX or DXR administration. Number of holes explored by animal was counted by an automatic counter during 5 min and considered as an exploratory activity.

Hyperactivity in mice was induced by D,L-amphetamine (2.5 mg/kg *sc*). MTX or DXR were injected *ip* 1 h, 7 and 14 days before amphetamine. The locomotor hyperactivity was measured during 30 min in the photoresistor actometers as described above.

Haloperidol-induced catalepsy was measured 1 h, 7 and 14 days after MTX or DXR administration. Mice received haloperidol (1 mg/kg *ip*) and 60 min later they were placed on two wood-

en bars (15 x 5 x 3.5 cm) every 15 min. Catalepsy scores were noted during 180 min as follows: forepaws on the bar for the period of time longer than 15 s – 0.5 point; hindlimbs on the bar for the period of time longer than 15 s – 0.5 point; forepaws and hindlimbs on two nearest bars for the period of time longer than 15 s – 2 points.

Pain sensivity in mice was measured in the hot test according to the method of E d d y and L e i m b a c h (6). Animals were placed individually on the metal plate heated to 56°C. The time (s) of appearance of the pain reaction (licking of the forepaws or jumping) was measured. Experiments were performed 1 h, 7 and 14 days after the administration of MTX or DXR.

Pain sensitivity was also measured by the writhing syndrome test of W i t k i n et al. (18). The test was performed in mice by the *ip* injection of 3% solution of acetic acid in a volume of 10 ml/kg 1 h, 7 and 14 days after MTX or DXR administration. The number of writhing episodes was counted during 30 min after the injection of 3% acetic acid.

Mice were decapitated 5 h or 14 days after the administration of MTX or DXR, their brains were immediately frozen. NA and DA content in the brain was measured spectrofluorimetrically, according to the method of C h a n g (4), with the modification of B r o d i e et al. (2). The content of neurotransmitters was expressed in μg/g of tissue.

The obtained results were presented as means ± SEM. Statistical significance of the differences was evaluated using. Student's *t*-test or exact Fischer's test.

RESULTS

MTX decreased body weight of mice during two weeks after the injection and results were significant in comparison to the initial values and to the control group. DXR did not produce the loss of body mass in comparison to initial values, but inhibited normal increase of body weight observed in the control animals during 2 weeks (Fig. 1). Neither MTX nor DXR had neurotoxic properties as they did not affect the motor coordination in the rota rod test. Both investigated substances decreased the locomotor activity as well as exploratory activity in mice examined 7 and 14 days after their administration (Tabs. 1 and 2). Moreover, the hyperactivity induced by amphetamine was inhibited by DXR and stimulated by MTX at 7^{th} and 14^{th} day (Tab. 3). In addition MTX diminished the cataleptogenic effect of haloperidol at 7^{th} and 14^{th} day, while DXR did not change its activity. The small prolongation of catalepsy was observed during the last two 15 min intervals 14 days after DXR (Fig. 2). All parameters described above were not changed 1 h after drugs administration.

DXR did not change the pain sensitivity in mice in the hot plate test. The small analgesic effect of the drug was observed only in a higher dose (10 mg/kg) 1 h after treatment in the writhing syndrome test. However, MTX showed analgesic properties in both tests performed 7 and 14 days after the injection and in writhing syndrome test also 1 h after the injection (Tab. 4).

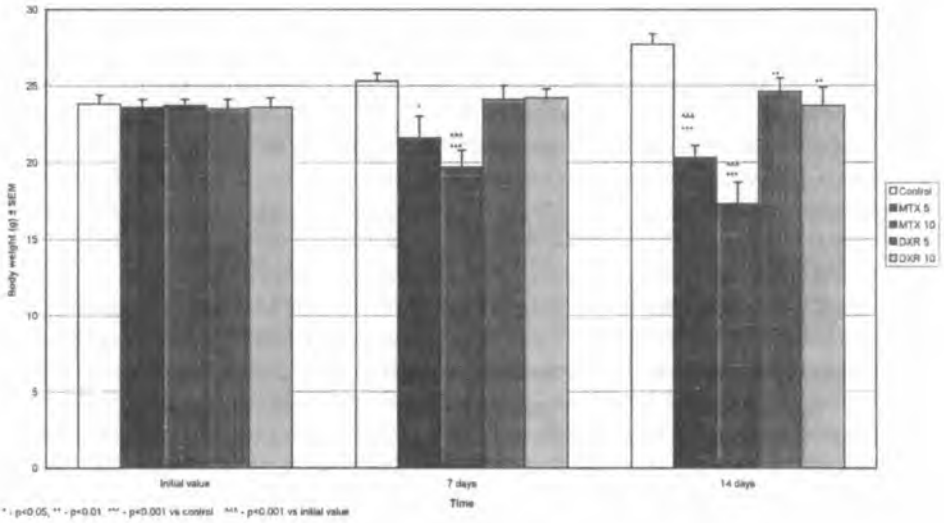

Fig. 1. The influence of MTX and DXR on the body weight of mice (n=12)

Table 1. The influence of MTX and DXR on the spontaneous locomotor activity in mice (n=12)

Compound	Dose mg/kg	Number of impulses ± SEM after time:		
		1 h	7 days	14 days
Control	solvent	335.8 ± 15.2	342.7 ± 9.8	339.4 ± 17.3***
MTX	5	302.8 ± 16.8	192.4 ± 26.3***	165.7 ± 19.5***
MTX	10	296.4 ± 24.3	138.3 ± 12.4***	106.2 ± 14.8***
DXR	5	311.5 ± 22.6	253.8 ± 21.5***	227.2 ± 16.4***
DXR	10	304.3 ± 23.7	229.6 ± 31.4***	197.3 ± 14.8***

*** p <0.01 vs control, Student's t-test.

In biochemical investigations it was found that both examined compounds, MTX and DXR, decreased brain NA content 5h and 14 days after their administration. The content of DA was strongly increased by MTX after 5 h and 14 days. DXR induced small enhancement of DA when administered in a higher dose (10 mg/kg) after 5 h. 14 days later the brain DA level returned to the control value (Tab. 5).

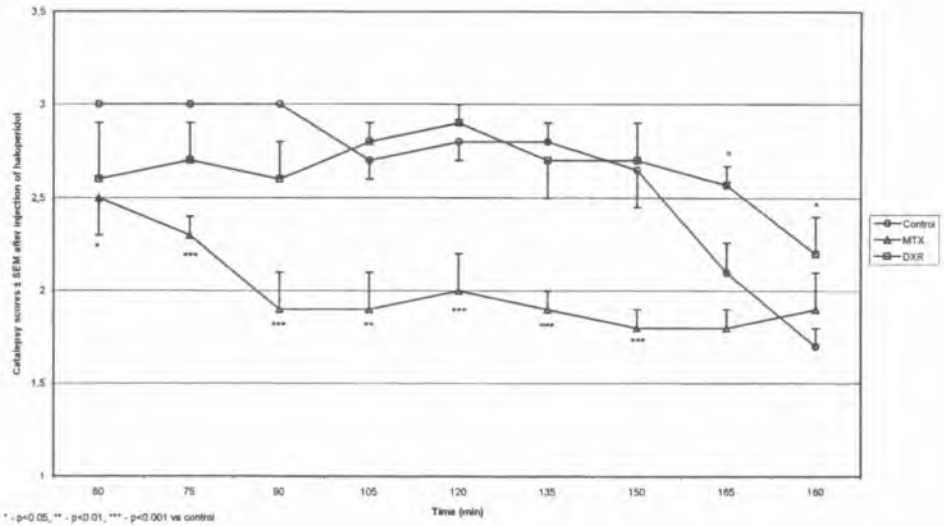

Fig. 2. The influence of MTX and DXR two weeks after the treatment on the haloperidol-induced catalepsy in mice (n=12)

Table 2. The influence of MTX and DXR on the exploratory activity in the hole-board test in mice (n=12)

Compound	Dose mg/kg	Number of penetrated holes ± SEM after time:		
		1 h	7 days	14 days
Control	solvent	36.5 ± 2.1	34.8 ± 1.8	35.7 ± 2.3
MTX	5	36.1 ± 2.5	28.6 ± 2.2*^	26.4 ± 3.3*^
MTX	10	31.4 ± 1.7	18.5 ± 3.1***^^	19.2 ± 2.7***^^
DXR	5	35.1 ± 2.7	30.1 ± 1.1*	31.0 ± 0.9
DXR	10	32.7 ± 3.3	22.9 ± 1.8***^	20.4 ± 2.1***^^

* $p < 0.5$; *** $p < 0.001$ vs control; ^ $p < 0.05$; ^^ $p < 0.01$ vs initial values, Student's t-test.

Table 3. The influence of MTX and DXR on the amphetamine induced locomotor hyperactivity in mice (n=12)

Compound	Dose mg/kg	Number of impulses ± SEM after time:		
		1 h	7 days	14 days
Control	solvent	493.4 ± 37.4	513.2 ± 34.9	544.3 ± 20.4
MTX	5	524.8 ± 31.6	779.5 ± 59.8**^^	880.4 ± 52.5***^^^

MTX	10	579.6 ± 24.1	954.2 ± 47.8***^^^	1022.3 ± 65.5***^^^
DXR	5	486.6 ± 40.5	401.7 ± 31.5*	438.6 ± 34.1
DXR	10	433.8 ± 151.4	379.7 ± 35.1***^	421.8 ± 32.7***^^

* p < 0.5; ** p<0.01 ,*** p<0.01 vs control; ^^ p < 0.01; ^^^ p< 0.001 vs initial values, Student's t-test.

Table 4. The influence of MTX and DXR on the pain sensivity in "hot plate"and "writhing syndrome" test in mice (n=12)

Compound	Dose mg/kg	Time reaction on thermal pain stimulus (s) ± SEM		
		1 h	7 days	14 days
Control	solvent	5.25 ± 0.48	5.15 ± 0.35	5.01 ± 0.27
MTX	5	6.94 ± 0.53	8.36 ± 0.72***	9.51 ± 0.84***
MTX	10	6.76 ± 0.43	9.52 ± 0.68***	11.31 ± 0.79***
DXR	5	5.50± 0.94	5.83 ± 0.43	5.92 ± 0.29
DXR	10	5.84 ± 0.72	6.28 ± 1.05	6.37 ± 1.01
		Number of writhing during 30 min ± SEM		
Control	solvent	25.2 ± 2.48	28.1 ± 3.35	27.1 ± 2.27
MTX	5	18.9 ± 1.53*	17.3 ± 2.72**	19.5 ± 2.84*
MTX	10	18.7 ± 1.43*	16.5 ± 2.68**	17.3 ± 1.79**
DXR	5	22.5 ± 2.94	26.8 ± 3.43	24.9 ± 2.29
DXR	10	18.2 ± 1.72*	25.2 ± 1.85	26.3 ± 1.72

* p<0.05; ** p<0.01 ,*** p<0.01 vs control, Student's t-test.

Table 5. The influence of MTX and DXR on the brain level of noradrenaline (NA) and dopamine (DA) in mice (n=12)

Compound	Dose mg/kg	Time (min.) after inj.	Brain level of neurotransmitters (μg/1 g of tissue) ± SEM	
			NA	DA
Control	solvent	–	0.165 ± 0.004	0.121 ± 0.005
MTX	10	5 h	0.137 ± 0.004***	0.179 ± 0.012***
MTX	10	14 days	0.121± 0.004***	0.165 ± 0.006***
DXR	10	5 h	0.149 ± 0.005*	0.136 ± 0.004*
DXR	10	14 days	0.132 ± 0.005***	0.133 ± 0.008

* p < 0.5; *** p <0.01 vs control, Student's t-test.

DISCUSSION

Both investigated oncostatics administered in a single high dose, comparable with doses used in clinical treatment of acute leukemia (5 or 10 mg/kg), showed the long-term toxicity, manifested in the decrease in the body mass of mice, but the action of MTX was more pronounced. This effect could be dependent on the influence on the epithelial tissue in the gastrointestinal tract and the diminution of the food consumption, but also the influence on the chemoreceptors of *area postrema* play an important role (5, 9). Similar results were observed by others after the administration of small doses (0.5 – 1.5 mg/kg) of rubidomycine and its new compounds during ten days (10). Our results indicate that the behavioral effects induced by both investigated drugs are connected with their central action. Neither MTX nor DXR showed the neurotoxic properties, as they did not affect the motor coordination in the rod rotating with 4 rpm. But they both strongly decreased the locomotor and exploratory activity during 14 days after their administration. These syndromes could be connected with the general, nonspecific depressive action on the CNS. This depressive effect could also be caused by the decrease of brain NA content induced by both compounds, which was found in our biochemical studies. Moreover, MTX possess the analgesic properties which were observed 1 h, 7 and 14 days after the treatment. This effect could have an additional meaning important in the treatment of terminal stages of neoplasms. DXR showed such activity only 1 h after the administration of a higher dose (10 mg/kg) and only in writhing syndrome test. Such a short, acute effect was observed by other authors in the action of most antracyclic antibiotics, and it seems to have no practical meaning (3, 10, 12 17).

DXR inconsiderably decreased the hyperactivity induced by amphetamine. This effect could be connected with general inhibition of locomotion. However, MTX strongly enhanced the action of amphetamine and this effect was present during 14 days after the administration. In addition MTX reduced the cataleptogenic properties of haloperidol, dopaminergic D-2 receptor antagonist, while DXR did not show such influence. The results of the biochemical studies confirmed that MTX affect brain dopaminergic system. The high increase of the level of DA was noted both 5 h and 14 days after the treatment. The enhancement of the DA content parallel to the behavioural syndromes of the stimulation of the dopaminergic activity, could be explained by the increase of the synthesis of this amine in brain tissue. However, our biochemical investigations were performed in the whole mice brain, using not very sensitive spectrofluorimetric methods. The results reflect only the static situation in the brain and cannot give the

evidence for the dynamic changes in vulnerable structures involved in the action of MTX. Anyway, the potentiation of the action of amphetamine and inhibition of action of haloperidol give the evidence that MTX increased the activity of brain dopaminergic system. These results could be induced by direct stimulation of dopaminergic D-2 receptors in the extrapyramidal system or indirectly by the increase of the synthesis of DA in brain tissue.

In conclusion, our results indicate that both investigated cytostatics, MTX and DXR, inhibited the increase of the body weight and suppressed the locomotor and exploratory activity of mice, but the action of MTX was more pronounced. Moreover, MTX had the clear analgesic properties, which could be noted during 14 days after single dose administration. In addition, MTX strongly stimulated brain dopaminergic system. DXR did not possess these properties, only the nonspecific depressive influence on the CNS could be observed.

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STRESZCZENIE

Doświadczenia przeprowadzono na białych myszach, samcach i samicach szczepu Albino-Swiss. Badano ośrodkowe działanie dwu powszechnie stosowanych klinicznie cytostatyków: antymetabolitu kwasu foliowego, metotreksatu (MTX) oraz doksorubicyny (DXR, adriablastyny), antybiotyku z grupy antracyklin. Obydwa leki stosowano dootrzewnowo (*ip*) w jednorazowych dawkach 5 i 10 mg/kg, które odpowiadały dawkom stosowanym w leczenu np. ostrych białaczek w okresie indukcji remisji. Doświadczenia behawioralne wykonywano po 1 godz., 7 i 14 dniach, a biochemiczne po 5 godz. i 14 dniach od zastosowania leków. Wykazano, że obydwa leki hamują przyrost masy ciała myszy, a MTX prowadzi nawet do jej obniżenia. Ponadto stwierdzono zahamowanie przez MTX i DXR ruchliwości spontanicznej i poznawczej zwierząt, przy braku działania neurotoksycznego w teście pręta obrotowego. MTX zwiększa aktywność układu dopaminergicznego, tj. nasila pobudzenie ruchowe wywołane amfetaminą oraz osłabia kataleptogenne działanie haloperidolu. W badaniach biochemicznych wykazano wzrost zawartości DA w mózgu, co na podstawie wyników badań behawioralnych może być tłumaczone nasileniem syntezy tej aminy. Ponadto MTX wykazuje działanie przeciwbólowe, utrzymujące się przez 14 dni w obu stosowanych testach. DXR działania takiego nie wywiera. Wykazano jedynie niespecyficzne działanie depresyjne DXR na OUN, manifestujące się zahamowaniem spontanicznej aktywności motorycznej i poznawczej.