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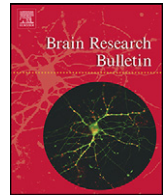
Research report

Inhibition of hippocampal cell proliferation by methotrexate in rats is not potentiated by the presence of a tumor

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ABSTRACT

Methotrexate is a widely used cytostatic in chemotherapy cocktails for the treatment of cancer but is associated with cognitive impairment. Previous animal studies indicated that methorexate decreases hippocampal cell proliferation, which might contribute to the observed cognitive impairment. However, clinical studies have shown that cognitive impairment can also be noticed in some cancer patients before any systemic treatment is initiated. We aim in the present study to discern whether hippocampal cell proliferation is negatively affected by tumor growth and if the presence of a tumor amplifies the effects of methotrexate.

Buffalo rats were subcutaneously injected with PBS or Morris Hepatoma 7777 cells to induce a tumor. Two weeks after this injection the animals received an intraperitoneal injection of methotrexate or saline. Three weeks later hippocampal cell proliferation was quantified using immunohistochemical staining.

Treatment with Morris Hepatoma 7777 cells decreased the number of proliferating cells as compared to control animals. An overall tumor effect was absent mainly because methotrexate treatment significantly decreased cell proliferation with no differences between animals with or without a tumor. Neither methotrexate nor the tumor induced pica behavior.

These findings indicate that although the presence of a tumor reduces hippocampal cell proliferation it does not affect the negative effect of methotrexate on this plasticity marker. Since sickness behavior is not induced by methotrexate or tumor presence it does not play a role in the development of cognitive deficits. This study further indicates that the effects of methotrexate on brain and behavior can be studied in healthy animals.

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1. Introduction

Chemotherapy is frequently used as adjuvant treatment strategy for cancer and is associated with cognitive impairment in part of the patients [3,28]. A number of animal studies have been carried out to explore the action of cytostatics on brain and behavior, and in these studies methotrexate (MTX), a cytostatic

agent used in an adjuvant chemotherapy cocktail, is often explored [6,14,18,21,22,29]. Our studies have shown that MTX has a negative effect on cognitive behavior and hippocampal cell proliferation, which was observed already shortly after treatment and lasted for several weeks [21,22]. This cell proliferation is part of the process of neurogenesis, which is thought to play an important role in learning and memory [8,12,13]. Therefore, a decrease in hippocampal cell proliferation may contribute to the cognitive impairment described above.

In animal models, the effect of cytostatics on cognitive behavior is generally examined in healthy animals, which diverges from the clinical situation. Furthermore, there are indications that a subgroup of cancer patients suffers from cognitive impairment before any systemic treatment is initiated [9,26,27]. There are currently no clear indications which neural principles might be involved in this impairment. Since we have shown in previous studies that MTX decreases hippocampal cell proliferation and impairs cogni-

tion in healthy rats [21,22], one of the aims of the present study was to validate our animal model by studying the effects of MTX in a tumor model. With this study, we tried to answer a number of questions: is the presence of a tumor negatively affecting hippocampal cell proliferation; is the effect of MTX on this proliferation actually potentiated by the tumor; and does MTX and/or tumor presence induce sickness behavior in tumor-bearing animals. Sickness behavior was studied since it is associated with decreased cognition [17]. Induction of proinflammatory cytokines can induce sickness behavior and both chemotherapeutic drugs and cancer cachexia are related to peripheral cytokine release [17,24].

We used an animal model of hepatoma, in which Buffalo rats were subcutaneously injected with Morris Hepatoma 7777 cells. After the appearance of cancer cachexia the animals were treated with a single, intraperitoneal injection of MTX. Sickness behavior was measured by the presence of pica behavior, which is the consumption of non-nutritive substances [2]. Cell proliferation was studied by measuring Ki-67 positive cells in hippocampal slices.

2. Methods

2.1. General

Twenty-eight adult (3 months of age) male Buffalo rats (average body weight at the start of the experiment 319 g ± 2.8 SEM) were obtained from Harlan (Zeist, the Netherlands). The animals were housed individually in clear Plexiglas cages on a layer of wood shavings with a fixed 12:12 h light:dark cycle (with lights on at 08.00 a.m.), and food and water *ad libitum*. Four experimental groups were used (PBS/saline, PBS/MTX, Morris Hepatoma 7777/saline, Morris Hepatoma 7777/MTX) each consisting of 7 rats and experiments started 3 weeks after arrival of the animals. The experiment was approved by the Animal Experimentation Committee of the University of Groningen and complied with the EC Council Directives.

Morris Hepatoma 7777 cells were obtained from LGC Ltd. (Middlesex, England) and grown according to the distributor's recommendations in standard DMEM medium, with 5% fetal calf serum, 4.5% glucose, at 37 °C, and 10% CO_2. The cells were harvested, centrifuged and transferred into sterile PBS and injected subcutaneously between the shoulder blades during a short-lasting anesthesia of isoflurane mixed with normal air. Cells were injected in a dosage of 7.5–10 million cells dissolved in 1 ml PBS per animal and the injection puncture was closed with a single suture. Control animals were injected according to the same procedure with PBS.

Body weight gain and food intake were measured daily and average food intake was calculated from day 2 until day 8 after the injection. MTX or saline was injected when the animal consumed 80% or less of its average food intake for 2 consecutive days. Half of the tumor-bearing animals and half of the control animals received an intraperitoneal injection of MTX (100 mg/kg, 100 mg/ml, Pharmachemie BV, Haarlem, the Netherlands), the remaining animals received saline. After the injection, animals received intraperitoneal injections of calcium leucovorin (10 mg/ml, Pharmachemie BV, Haarlem, the Netherlands), which is clinically used as a so-called rescue therapy in combination with the cytotoxic agent. Pilot studies revealed that leucovorin itself does not have an effect on cell proliferation and that high-dose MTX without leucovorin is lethal due to severe diarrhea and weight loss. This rescue therapy is based on the fact that leucovorin is a tetrahydrofolate (THFA) that does not require activation by THFA reductase. Tetrahydrofolate is a cofactor in DNA synthesis; MTX is an inhibitor of the enzyme THFA reductase and depletes the pool of tetrahydrofolates [7,10]. The rescue therapy of leucovorin was administered in a protocol similar to the application in patients. Eighteen hours after the injection of MTX, leucovorin was administered in a concentration that was 8% of the injected MTX dosage; at 26, 42, and 50 h the administered concentration was reduced to 4%.

2.2. Pica behavior

Kaolin pellets were made according to a protocol of Vera et al. [25]. In brief, kaolin powder, gum Arabic, and carmine (all substances were purchased from Sigma Aldrich, Lyon, France) were mixed in a proportion of 98.5%, 1%, and 0.5% respectively. Carmine was added to the mixture because it colors faeces pink. Distilled water was added to the mixture to create a thick pasta which was molded into pellets and air dried for 48 h at room temperature. The pellets were placed in the cage directly after the injection of MTX or saline in cachexic and control animals for 1 week to see whether MTX or anorexia cachexia causes pica behavior. The pink faeces was removed from the cage daily and weighed, to achieve an estimation of the pica behavior.

2.3. Effect of MTX and tumor growth on hippocampal cell proliferation

The animals were sacrificed 3 weeks after the injection with saline or MTX through transcardial perfusion with saline followed by 4% paraformaldehyde and the tumors were removed and weighed. Brains were removed and placed in 30% sucrose solution at 4 °C. Microtome sections of the hippocampus (30 μm) were stored at −20 °C in 30% ethyleneglycol/30% glucose in PBS solution until immunohistochemical staining.

From the serial sections, every sixth section from each animal was selected and immunocytochemically stained for Ki-67 using a slightly adapted standard protocol [11]. In brief, free-floating sections were pre-treated with 0.4% H_2O_2 for 30 min, to stop endogenous peroxidase activity. Non-specific binding of immunoreagents was blocked with 3% normal goat serum (Zymed, San Francisco, CA, USA). Subsequently, sections were incubated with mouse-anti-Ki-67 (1:200, Novocastra, Newcastle upon Tyne, UK), for 48 h at 4 °C. After a second blocking step, sections were incubated with a biotinylated secondary antibody (1:400, goat-anti-mouse, Jackson, Wet Grove, PA, USA) for 2 h at room temperature. This was followed by incubation in an avidin biotinylated peroxidase complex (1:400, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Labeled cells were visualized with 0.15 mg/ml diaminobenzidine and 0.003% H_2O_2 solution.

After mounting the sections onto glass slides for microscopic analysis, sections were counterstained with a Mayer-haematox solution for 30 s. Counting of Ki-67 positive cells in both hemispheres of the dentate gyrus was performed under a light microscope with a magnification of 400×. Counting was performed in the subgranular layer of the dentate gyrus and counts in both blades were summed. The border of the area that was quantified was defined as the subgranular layer having a thickness of two cell diameters. All cells were counted in the subgranular layer of the dentate gyrus from top to bottom of the 30 μm thick sections. Because every sixth section of the brain was stained, with a total of 18 slices per animal, the amount of positive cells was multiplied by 6 to get the estimated total amount of Ki-67 positive cells in the hippocampus.

2.4. Statistics

Body weight and food intake were analyzed using two-way repeated measure ANOVA. Significant differences between the different days for body weight and food intake was measured with an independent-sample T-test when the two-way repeated measure ANOVA was significant. Light-microscopic counts of Ki-67 positive cells were analyzed using two-way ANOVA. In all tests PBS/Morris Hepatoma 7777 and saline/MTX were used as between-subject variables. For all statistical tests, a probability value less than 0.05 was considered to be statistically significant.

3. Results

3.1. The effect of Morris Hepatoma 7777 cells and MTX on body weight gain

Body weight of all animals was measured daily and expressed as percentage of the body weight on the day of the injection with PBS or Morris Hepatoma 7777 in Fig. 1. The decrease in body weight gain in the animals treated with Morris Hepatoma 7777 indicates anorexia cachexia. After the onset of anorexia cachexia, which was also determined by a consistent reduction in food intake, the animals received MTX or saline.
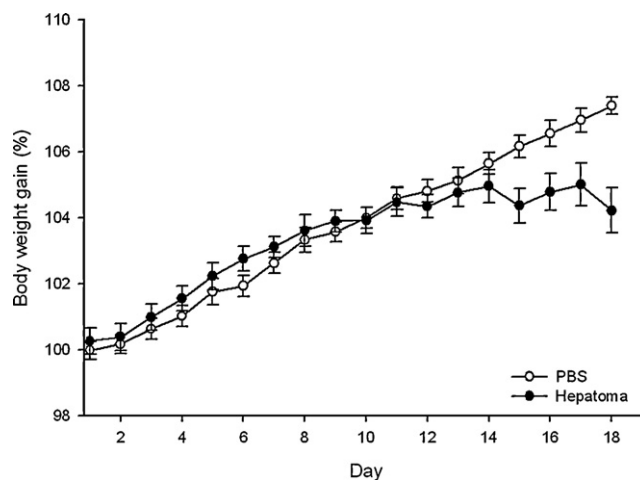

Fig. 1. Body weight gain after treatment with PBS (○) or Morris Hepatoma 7777 (●). The decrease in body weight gain in animals treated with Morris Hepatoma 7777 indicates the onset of anorexia cachexia.
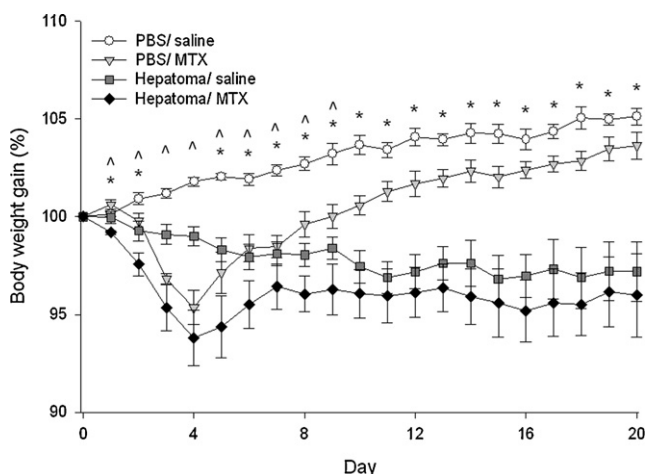
Fig. 2. Body weight gain after intraperitoneal administration of saline or MTX. The different groups are PBS/saline (○), PBS/MTX (▽), Morris Hepatoma 7777/saline (■), and Morris Hepatoma 7777/MTX (♦). Body weight before the injection (given on day 0) is expressed as 100%; bars represent standard error of the mean. A main effect was found between treatment with PBS and Morris Hepatoma 7777 and between treatment with saline and MTX. No interaction effect between PBS/Morris Hepatoma 7777 and saline/MTX was found. The * and ^ represent a significant difference between animals treated with PBS or Morris Hepatoma 7777, and animals treated with saline or MTX respectively.

Body weight of all animals after treatment with MTX or saline was measured daily and expressed as percentage of the body weight on the day of the injection. Fig. 2 shows that body weight decreased in animals treated with MTX. Animals treated with PBS/MTX started to regain body weight from day 5, whereas the tumor-bearing animals treated with MTX did not. The body weight in tumor-bearing animals treated with saline also decreased during the experiment. A main effect was found between treatment with PBS and Morris Hepatoma 7777 ($F(1.24) = 50.737$, $P < 0.001$). When analyzed with an independent-sample T-test a significant difference was seen between animals treated with PBS and Morris Hepatoma 7777 from day 1 until day 20 ($P < 0.05$), with the exception of days 3 and 4 which was caused by a decrease in body weight gain due to MTX treatment. A main effect was also found between treatment with saline and MTX ($F(1,24) = 10.593$, $P < 0.005$). When analyzed with an independent-sample T-test a significant difference was seen between animals treated with saline and MTX from day 1 until day 9 ($P < 0.05$). No interaction effect between PBS/Morris Hepatoma 7777 and saline/MTX was found.

3.2. The effect of Morris Hepatoma 7777 cells and MTX on food intake

Food intake of all animals was measured daily following MTX or saline administration and is shown in Fig. 3. The animals treated with MTX decreased food intake directly after the injection. However, in animals treated with PBS/MTX food intake reached the level of the animals treated with PBS/saline after day 6, whereas food intake of animals treated with Morris Hepatoma 7777/MTX remained at the level of the animals treated with Morris Hepatoma 7777/saline. A main effect was found between treatment with PBS and Morris Hepatoma 7777 ($F(1,24) = 39.463$, $P < 0.001$). When analyzed with an independent-sample T-test a significant difference was seen between animals treated with PBS and Morris Hepatoma 7777 from day 0 until day 20 ($P < 0.05$), with the exception of days 3 and 4 which was caused by a decrease in body weight gain due to MTX treatment. A main effect was also found between treatment with saline and MTX ($F(1,24) = 4.402$, $P < 0.05$). When analyzed with
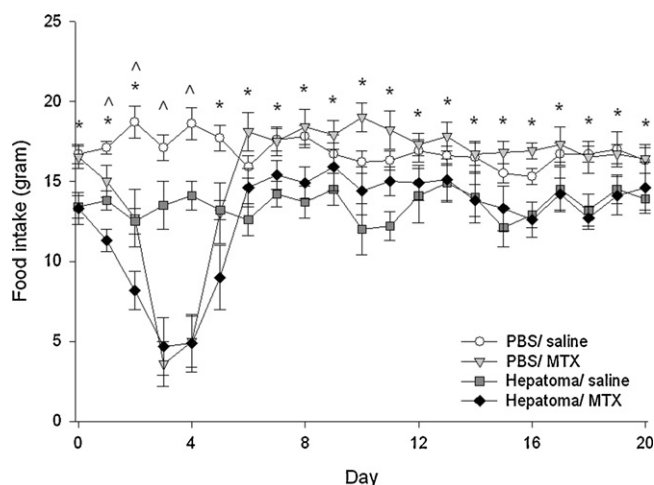

Fig. 3. Food intake after intraperitoneal administration of saline or MTX. The different groups are PBS/saline (○), PBS/MTX (▽), Morris Hepatoma 7777/saline (■), and Morris Hepatoma 7777/MTX (♦). Data are represented as mean ± standard of the mean. A main effect was found between treatment with PBS and Morris Hepatoma 7777 and between treatment with saline and MTX. No interaction effect between PBS/Morris Hepatoma 7777 and saline/MTX was found. The * and ^ represent a significant difference between animals treated with PBS or Morris Hepatoma 7777, and animals treated with saline or MTX respectively.

an independent-sample T-test a significant difference was seen between animals treated with saline and MTX from day 1 until day 5 ($P < 0.05$). No interaction effect between PBS/Morris Hepatoma 7777 and saline/MTX was found.

3.3. Pica behavior

Kaolin pellets were placed in the cage of the animals directly after the injection with MTX or saline to study the effect on pica behavior as a measure of sickness behavior. Carmine was added to the pellets, so the consumption of kaolin could be measured by the presence of pink faeces. The amount of pink faeces was low in all groups (average amount for all groups together $0.3 \, g \pm 0.7$ SEM), with no significant differences between the groups (data not shown).

3.4. Effect of MTX and tumor growth on hippocampal cell proliferation

Three weeks after treatment with MTX, the animals were sacrificed and the tumors were removed and weighed. There was no significant difference in the weight of the tumor between saline treated animals and animals treated with MTX. The relative tumor weight per total body weight was $1.65\% \pm 0.59$ SEM for animals treated with saline, and $1.90\% \pm 0.76$ SEM for animals treated with MTX. The absolute tumor weight was 5.26 ± 1.77 SEM for animals treated with saline, and 5.75 ± 2.28 SEM for animals treated with MTX.

Ki-67 positive cells in microtome sections of the hippocampus were visualized and counted (Fig. 4). The total number of Ki-67 positive cells were 1114.5 ± 304.5 SEM for animals treated with PBS/saline; 470.4 ± 106.2 SEM for animals treated with PBS/MTX; 752.4 ± 95.3 SEM for animals treated with Morris Hepatoma 7777/saline; and 440.4 ± 164.8 SEM for animals treated with Morris Hepatoma 7777/MTX. A main effect was found between treatment with saline and MTX ($F(1,24) = 10.117$, $P < 0.01$). No main effect was found between treatment with PBS and Morris Hepatoma 7777. However, this is most likely the result from the large decrease in hippocampal cell proliferation after MTX treatment which may indicate a ceiling effect. When compared individually with a one-
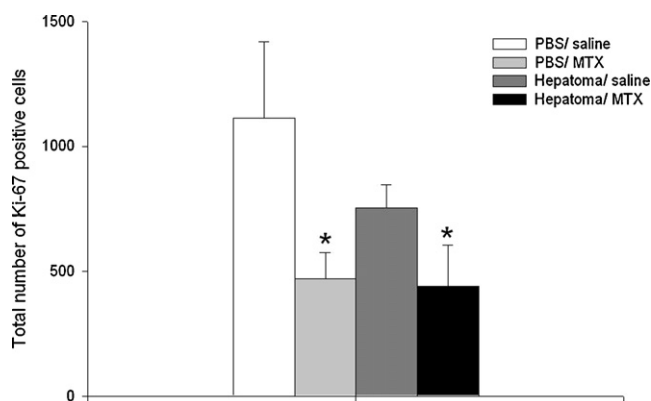
Fig. 4. Total number of Ki-67 positive cells in the hippocampus of animals treated with PBS/saline (□), PBS/MTX (▨), Morris Hepatoma 7777/saline (▨), and Morris Hepatoma 7777/MTX (■). Data are represented as mean with standard error of the mean. A main effect was found between treatment with saline and MTX (represented with *). No main effect was found between treatment with PBS and Morris Hepatoma 7777. Since this is mainly caused by the fact that MTX treatment causes a similar decrease in cell proliferation in animals with and without a tumor, we noticed that Morris Hepatoma 7777 administration did cause a significant inhibition of hippocampal cell proliferation when compared to the PBS/saline control group. No interaction effect between PBS/Morris Hepatoma 7777 and saline/MTX was found.

way ANOVA, a significant effect was seen between the different groups ($F(3,18) = 4.036$, $P < 0.05$). Test for contrast revealed a significant different between animals treated with PBS/saline and Morris Hepatoma 7777/saline ($P < 0.001$). No interaction effect between PBS/Morris Hepatoma 7777 and saline/MTX was found.

4. Discussion

We explored in this paper the effect of MTX on hippocampal cell proliferation in a tumor model. MTX significantly reduced the total number of Ki-67 positive cells in the hippocampus compared to control animals, indicating a decreased amount of proliferating cells. The negative effect of MTX on hippocampal cell proliferation was expected since our previous studies also showed a decrease in the total number of Ki-67 positive cells after treatment with MTX [21,22]. MTX is a dihydrofolate reductase inhibitor and has its effect on cell death by inhibiting the conversion of folic acid into tetrahydrofolate thereby inhibiting the synthesis of purine and thymidine [15]. In case the effects of MTX are mediated by a direct action of MTX in the brain, a sufficient amount of MTX penetrates the brain to have this effect on hippocampal cell proliferation as well.

Cognitive impairment is not only described after treatment with chemotherapy [1,3,16,20,23,28], but also in the period between diagnosis and systemic treatment [9,26,27]. The presence of a tumor in our study did appear to decrease the number of proliferating cells in the hippocampus, suggesting that this may contribute to the cognitive impairment observed in cancer patients before any treatment is initiated. In patients additional explanations for this early cognitive impairment can be found in diagnosis related emotional stress, or DNA damage and/or deficiencies in DNA repair mechanisms [9,26,27] with the latter two being linked both to the development of cancer and neurodegenerative disorders. The presence of a tumor, however, did not further enhance the negative effects of MTX on hippocampal proliferation. This finding indicates that the effects of adjuvant chemotherapy on hippocampal cell proliferation as observed in healthy animals can be extrapolated to tumor-bearing individuals.

Besides the effect of MTX and cancer on hippocampal cell proliferation, we also explored the effect on body weight gain and sickness behavior. The tumor-bearing animals in our study showed clear signs of cancer cachexia, which can be seen in the arrest or lowering of body weight gain while food intake remained stable but at a lower level compared to control animals. Anorexia cachexia is a phenomenon frequently observed in cancer patients and is associated with the early stages of the disease, serving as a diagnosis tool, as well as with the terminal stages of cancer. This side effect of cancer is described as both the loss of adipose tissue and skeletal muscle mass resulting in a high co-morbidity factor in patients. Anorexia cachexia induces metabolic changes as well, such as altered carbohydrate and protein metabolism [24]. This anorexia cachexia is, however, not related to sickness behavior since cancer cachexia did not coincide with an increase in pica behavior. Neither did MTX induce sickness behavior. MTX did reduce food intake but this was caused by diarrhea as a consequence of damage to the cells of the intestinal tract which is a side effect of MTX [7,10]. Pica behavior after MTX is also not described in the literature although other cytostatics are associated with this sickness behavior. Cisplatin is especially known to induce pica behavior [2,4,5,19,30], but also cyclophosphamide, actinomycin D, and 5-fluorouracil are associated with sickness behavior [30].

In general, we can conclude that animals treated with MTX showed a decrease in hippocampal cell proliferation, which possibly contributes to the cognitive impairment seen in some cancer patients after adjuvant chemotherapy. Since we did not find an interaction effect between MTX and cancer on hippocampal cell proliferation, our animal model in which we treat healthy animals with MTX is a validated model to test potential mechanisms that may contribute to the cognitive impairment seen after adjuvant chemotherapy treatment.

Conflicts of interest

The authors declare that they have no competing financial interests.

Acknowledgments

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