Intraneoplastic injection of methotrexate for experimental brain-tumor chemotherapy

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The retention and distribution of tritiated methotrexate (MTX-3H) after direct intracerebral or intraneoplastic injection were studied in mice bearing subcutaneous or intracerebral ependymoblastomas. After intracerebral injection of MTX-3H in nontumor-bearing animals, a large amount of the drug was retained in the head, much more than could have been retained after systemic administration, and there was rapid spreading of the drug through the ipsilateral hemisphere. Intraneoplastic injection of subcutaneous and intracerebral tumors produced rapid spreading of the drug through the tumors. Initially, the drug was mainly in the interstitial fluid of the tumors followed by earlier cellular uptake than was seen after intravenous injection. Even though the distribution of the drug in the intracerebral tumors was not uniform, and some intracranial tumor deposits contained less radioactivity than areas closer to the site of injection, intraneoplastic injection may have advantages for brain-tumor chemotherapy. However, further experimental study is necessary before clinical application can be recommended, especially evaluation of neurotoxicity after intracerebral, intraneoplastic injection of MTX or other chemotherapeutic agents.

Key Words • brain tumor • chemotherapy • methotrexate • intraneoplastic injection

Oral, intravenous, or intraarterial administration of methotrexate (MTX) has not been effective for the chemotherapy of patients with brain tumors. Similarly, animals with intracerebral implants of glial or other tumors have not responded well to parenteral MTX. Other routes of administration of MTX such as the intrathecal route have been used with varying results. For example, in patients with meningeal leukemia, it has long been recognized that the intrathecal route of MTX administration is more effective than the systemic route. Recently, Ushio, et al., showed that there was a much higher uptake of tritiated methotrexate (MTX-3H) after intrathecal injection than after intravenous injection in intracerebral implants of transplantable mouse gliomas. Direct intraneoplastic injection of MTX has also been studied in patients with brain tumors but the results have not been favorable. In our laboratory, we initially showed autoradiographically that intravenously administered MTX-3H failed to reach all areas of an invasive experimental intracranial mouse ependymoblastoma up to 1 hour after injection. Subsequently we showed that non-uniform uptake was still present even after an interval of 7 days from the time of intra-
venous injection, and that this experimental glioma even when grown subcutaneously accumulated only small amounts of intravenously injected MTX. The amount accumulated by the tumor was much less than that taken up by tissues such as liver or kidney. In addition, the uptake of this drug by the tumor was much less than the uptake of several other agents, including radioiodinated human serum albumin.

For these reasons, we have been studying the direct intraneoplastic method of administering MTX for brain-tumor chemotherapy. Although the intrathecal route has been studied clinically and experimentally, and the direct intraneoplastic route has been used clinically for the administration of MTX, there has not been any previous evaluation of this technique in experimental brain tumors. Knowledge of the behavior of MTX following direct intraneoplastic injection in experimental brain tumors would be useful for the design of effective dosage schedules for clinical brain-tumor chemotherapy with this technique.

Materials and Methods

Materials

We used a mouse ependymoblastoma obtained in 1963 and maintained in our laboratory by serial subcutaneous transplantation every 2 weeks. The tumor was originally induced in mouse brain with intracerebrally implanted methylcholanthrene. The experimental animals were C57BL/6J female mice, weighing 16 to 18 gm.*

Tumor Implantation

Subcutaneous tumors were grown by implanting with a forceps 25-mg pieces of the mouse ependymoblastoma into the subcutaneous tissues of the right lower abdominal quadrant of the mice. These tumors grew to approximately 1 cu cm by about 20 days after implantation and were then used for the direct intraneoplastic MTX injection experiments. Intracerebral tumors were grown by injecting 3 μl of a tumor cell suspension containing 100,000 cells/μl into the right frontal region by means of a specially designed stereotaxic frame. The methods used for preparing the tumor cell suspension and for injecting it intracerebrally have been described in detail previously. Mice received MTX-3H injections 20 days after tumor implantation.

Tracer

We obtained MTX-3H, nominally labeled with tritium in the 3' and 5' positions of the phenyl ring, at a specific gravity of 13 curies/millimol. Radiochemical purity was 98% in both systems, assessed by paper chromatography by the suppliers in two systems using 0.5% sodium carbonate and n-butanol:pyridine:water (1:1:1). Further purification was performed in our laboratory by spotting the material on Whatman 3 MM filter paper. The chromatogram was developed with 0.5% sodium carbonate for 3 hours and then dried. A strip the length of the chromatogram was cut from the middle portion, counted in a chromatograph scanner and the MTX-3H identified. The scanned strip was then realigned with the remaining two pieces of the chromatogram paper and the appropriate sections containing MTX-3H were cut from the chromatogram paper. MTX-3H was then eluted from these sections with 30 ml of distilled H2O (pH 8.4 adjusted with NaOH), placed in a flask, and shell frozen in dry ice and acetone. The flask was then placed on a continuous freeze dryer, and freeze-dried for 18 hours; the residue was then taken up in 0.76 ml of distilled H2O. An aliquot of the purified MTX-3H was then rechromatographed on Whatman No. 1 paper and developed with 0.5% sodium carbonate. Repeat radiochromatogram scanning showed only one peak containing greater than 98% of the radioactivity.

Experimental Protocol

Retention of MTX-3H After Direct Intracerebral Injection. Sixty nontumor-bearing mice received a 3-μl intracerebral injection containing 2 microcuries of MTX-3H.

*The mice were obtained from Jackson Laboratory, Bar Harbor, Maine.
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The injections were made into the right frontal region through a No. 30 needle by means of the stereotaxic frame previously described. Twelve mice were killed in ether, and then given the intracerebral injection post mortem to serve as "0" time standards. Of the remaining mice, groups of eight were killed by decapitation at the following time intervals after intracerebral injection of MTX-3H: 2 minutes; 10 minutes; 1 hour; 4 hours; 24 hours; and 72 hours. At all time intervals, the radioactivity concentration in the whole head and in samples of blood and kidney was assayed as described below.

Autoradiographic Distribution of MTX-3H After Direct Intracerebral and Intraneoplastic Injection. Eight mice with subcutaneous tumors were given a 3-µl intraneoplastic injection containing 2 microcuries of MTX-3H directly into the center of the tumor mass. The animals were then killed by decapitation and prepared for autoradiography as described below. Four animals were studied at 2 minutes and four at 1 hour after injection.

The stereotaxic frame and No. 30 needle were used for intracerebral injections in eight nontumor-bearing animals and for intraneoplastic injections in 24 animals bearing intracerebral tumors. All these animals received 3-µl injections containing 2 microcuries of MTX-3H. Four nontumor-bearing mice were prepared for autoradiography at 2 minutes after intracerebral injection and four at 1 hour. Four mice with brain tumors were prepared for autoradiography at each of the following times after intracerebral intraneoplastic injection: 2 minutes; 10 minutes; 1 hour; 4 hours; 24 hours; and 72 hours.

Sample Preparation for Combustion

Tissue samples containing MTX-3H and ranging in weight from 50 to 200 mg were placed in ashless filter-paper baskets made from 2.1-cm diameter Whatman No. 541 filter-paper circles. The samples were kept in the vials until just before combustion, at which time they were removed from the vials, rolled in a 9.0-cm diameter Whatman No. 42 ashless filter-paper circle, and compressed into pellets in a pellet press to ensure maximum recovery and uniformity of burning. At the time of sacrifice, blood samples were placed directly on filter-paper pellets in preweighed vials that were then reweighed.

For determining MTX-3H concentration in the entire head, the head was first digested in 10 ml of 5 normal potassium hydroxide (5N KOH) in a water bath at 50 °C. After complete digestion a 0.1 ml aliquot was then placed on filter paper and prepared for combustion as above.

Sample Combustion and Counting

The samples were oxidized in a sample oxidizer. Tritiated water was collected in a vial of scintillation fluid made up of naphthalene, 100.0 gm; 2, 5-diphenyloxazole (PPO), 5.0 gm; dimethyl p-bis[2-(5-phenyloxazolyl)]-benzene (POPOP), 0.3 gm; dioxane, 720 ml; toluene, 135 ml; and absolute methanol, 45 ml. This cocktail gave a counting efficiency of 24% to 28%.

The recovery efficiency of the automated combustion technique was determined at regular intervals. A standard solution was made by putting 20 microcuries of 3H tracer in 100 ml of water from which 0.1 ml aliquots were pipetted into each of three vials filled with scintillation fluid and onto each of five filter-paper pellets. The filter-paper pellets were then oxidized and the radioactive products were collected in the usual manner. The radioactivity recovered in the oxidized pellets was then compared with that placed directly in the scintillation fluid and the recovery was found to be about 92% to 96%. There was a carryover of radioactivity from one combusted sample to the next of approximately 2%. To prevent this, a blank filter-paper pellet was oxidized between radioactive tissue samples. The 3H standard solutions were counted with the tissue samples at 4 °C in a liquid scintillation counter,™ by the channels ratio method. A minimum of 10,000 counts were recorded for each sample.

Calculations

The radioactivity in the tissues is expressed as the percentage mean body concentration

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™Unilux II liquid scintillation counter manufactured by Nuclear-Chicago, 2954 Peachtree Road, N.E., Atlanta, Georgia 30319.
Fig. 1. Graph shows the distribution of MTX-3H in whole head (black squares), blood (triangles), and kidney (circles) of nontumor-bearing animals at various time intervals following the intracerebral injection of MTX-3H. The values for the initial time “0” determinations are the means of 12 mice and the other values are the means of eight mice. This a log-log plot with the retention (whole head) or uptake (blood, kidney) in % mean body concentration of MTX-3H along the ordinate and the time in hours along the abscissa. The standard deviations are shown as vertical bars.

(% MBC). This value is a ratio of the dpm/gm tissue to the dpm injected per gm animal:

\[
\text{dpm/gm tissue} \times \frac{\text{dpm injected/gm animal}}{100} = \% \text{MBC.}
\]

Expressed this way, the % MBC of a radiotracer that is uniformly distributed and not excreted is 100% in all tissues. To determine the dpm injected per gm animal, an aliquot of injected tracer was placed on filter-paper pellets, combusted, and counted. All calculations were done on a Sigma 5 computer by the Medical Computing Department.

Autoradiography

Since MTX-3H is a diffusible tracer, special techniques must be used for autoradiography to prevent leaching or movement of the tracer in the tissues during processing.

The techniques used here have been described in detail previously and consist of the dry mounting of frozen, dried 2-μ sections on slides coated with Kodak NTB3 emulsion. The exposure times ranged from 30 to 60 days. After developing and fixing, the autoradiographs were stained with methyl green pyronin Y.

Results

The retention of MTX-3H following direct intracerebral injection into normal mice is shown in Fig. 1. The “0” time % MBC in the whole head was 710.8. By 2 minutes after injection, the retention in the head had dropped sharply to 311.4, at 1 hour, to 124.5, and at 72 hours, to only 8.0. Figure 1 also shows the rapid entrance of MTX-3H into the blood stream and the extremely rapid accumulation in the kidney. In fact, by 10 minutes the % MBC in the kidney exceeded that in the head. After 1 hour the % MBC in the kidney...
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Fig. 2. Autoradiographs of normal mouse brain 2 minutes and 1 hour after intracerebral injection of MTX-3H. A to D are sections from an animal studied at 2 minutes after injection, and E to H are from an animal studied at 1 hour. There has been a marked decrease in the amount of blackening between 2 minutes and 1 hour after injection. The arrows mark the site of injection. The sections are from the right cerebral hemisphere with A and E the most superior and D and H the most inferior in each animal. × 6.

remained high while the % MBC in the blood and head continued to fall.

The autoradiographs of normal brain and tumor-bearing brain also provided evidence of the rapid disappearance of MTX-3H from the head. Figures 2 and 3 show a progressive diminution in the degree of blackening in the center of the injection site at increasing time intervals from the time of injection. The retention in the tumor-bearing brain (Fig. 3) was greater than in the normal brain (Fig. 2). At the early times the tracer appeared to be retained in a spherical or fusiform pattern that was apparent on gross examination of multiple horizontal sections from above downward (Fig. 2).

In the tumors the tracer rapidly spread from the site of injection and streamed through the interstitial fluid. Neoplastic cells in the path of the tracer became labeled even at the early times (Fig. 4). The MTX-3H was rapidly reabsorbed into the blood stream through cerebral blood vessels (Fig. 5) and through vessels in the tumors. The choroid plexus also appeared to be involved in the reabsorption process since there were usually many grains associated with the blood vessels in the fronds of choroid plexus tissue (Fig. 6).

The rapid permeation of MTX-3H through the normal or tumor-bearing brain was a remarkable feature demonstrated by the autoradiographs. Even at 2 minutes after injection the drug had spread about 3 mm from the site of injection (Figs. 2, 3, and 4) and by 1 hour the drug had spread about 6 mm from the site of injection. Due to drug reabsorption and spreading, estimation of degree of spreading could only be made microscopically. In some animals at 1 hour the drug was present throughout the cerebral hemisphere ipsilateral to the injection. The hippocampus and the ventricular wall sometimes appeared to retard the spread. Neurons and glial cells in the immediate path of the tracer showed
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FIG. 3. Autoradiographs of tumor-bearing mouse brain 2 minutes (A and B) and 1 hour (C and D) after intracerebral injection of MTX-3H. Although there has been a progressive diminution with time in the intensity of blackening at the site of injection, there has been spreading of radioactivity from the site of injection. The arrows mark the site of injection. The sections are from the right cerebral hemisphere with A and C more superior to B and D. × 6.

 variability in labeling, with some of the cells heavily labeled (Fig. 7). At sites distant from the injection the spread appeared to be interstitial but this was impossible to prove by light microscopy. In some animals there was evidence of tracer leakage from the injection site to the subarachnoid space with subsequent distribution through the subarachnoid space.

In the subcutaneous tumors the spread from the site of injection was extremely rapid. The drug spread quickly through the interstitial fluid and by 1 hour much of the radioactivity had already disappeared. Grains were seen around blood vessels presumably due to the reabsorption of MTX-3H into the blood stream. At 2 minutes there were some heavily labeled neoplastic cells adjacent to the injection site, but most of the radioactivity was interstitial, whereas at 1 hour almost all of the neoplastic cells were heavily labeled.

FIG. 4. Autoradiograph of intracerebral ependymoblastoma 2 minutes after intraneoplastic injection of MTX-3H. There are more grains at the top, which was closer to the site of injection. The grains are seen streaming through the interstitial space. Numerous neoplastic cells are labeled. × 560.

FIG. 5. Autoradiograph of brain 2 minutes after intraneoplastic injection of MTX-3H. The area shown was adjacent to intracerebral tumor and to the site of injection of MTX-3H. The grains are streaming through the cerebral tissue and are being reabsorbed into the large blood vessel seen at the lower left. × 360.

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Although considerable radioactivity remained in the interstitial fluid.

The intracerebral tumors showed similar features; the distribution was mainly interstitial at 2 minutes (Fig. 4) and mainly intracellular at the later times (Fig. 8 left). Significant cellular labeling persisted for 72 hours although the amount of radioactivity present was much less than at the earlier times. The distribution of MTX-3H in the intracerebral tumors showed some important differences from that in the subcutaneous tumors. Some areas of neoplastic spread within the brain continued to be less well labeled than the neoplastic cells growing within the central mass of the intracerebral tumors (Fig. 8 right). For example, there was less labeling of intraventricular neoplastic cells.

Discussion

Although MTX has been an effective chemotherapeutic agent for some types of leukemia, and for certain solid tumors such as the choriocarcinoma, it has not been effective in the treatment of most primary brain tumors. The resistance of brain tumors to MTX may be due in part to the lack of availability of MTX to all the proliferating intracerebral tumor cells. Our previous studies of intravenously administered MTX-3H in mice with intracerebral implants of the ependymoblastoma showed that the central tumor mass was heavily labeled but the tumor cells infiltrating the surrounding brain and those growing intraventricularly were poorly labeled. It was hoped that direct intraneoplastic injection of the drug would provide higher MTX concentrations in all intracerebral neoplastic deposits. Although there are no previous studies of directly injected MTX in experimental brain tumors, it has been shown recently in mouse brain tumors that intrathecal injection produced higher tumor uptake of MTX-3H than intravenous injection.

It was felt that knowledge of the behavior of MTX in mice after intracerebral injection might improve the results with this route in patients, which so far have been poor.

The present study showed that an extremely large amount of the injected MTX-3H remained in the head after intracerebral injection even though much was absorbed into the bloodstream. Comparison of the
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Fig. 8. Autoradiograph of intracerebral tumor 72 hours after intraneoplastic injection of MTX-3H. X 560. Left: The field shown is adjacent to the site of injection into the central mass of the tumor, and most of the neoplastic cells are labeled. Almost all the grains are present over the neoplastic cells with very little in interstitial sites as compared with Fig. 4. Right: Field shown is from an area remote from the site of injection, and at the periphery of the intracerebral tumor. In contrast to those at left, most neoplastic cells are not labeled and those that are labeled have only 1 or 2 grains.

Retention of MTX-3H in the nontumor-bearing head after intracerebral injection with the amount retained in the normal brain or subcutaneous ependymoblastoma after intravenous injection in our former study reveals that much more MTX-3H is retained after direct intracerebral injection, especially during the first hour (Table 1). For example, at 10 minutes the % MBC in the head after intracerebral injection was 233.2, whereas after intravenous injection in normal brain it was only 3.6, and in subcutaneous tumor only 25.4. At 1 hour the corresponding values were 124.5 after intracerebral injection and 5.1 and 19.0 after intravenous injection. Thus, by the intracerebral route it was possible to deliver and have retained in the brain much higher amounts of MTX-3H than by the intravenous route. This difference would have been even greater if retention in the tumor-bearing head had been measured by liquid scintillation counting, since autoradiography revealed much greater retention in tumor-bearing brain than in normal brain (Figs. 2 and 3).

The rapid spread of MTX-3H through the normal brain in the ipsilateral hemisphere was unexpected. Methotrexate is a water-soluble ionized compound of small molecular weight, and when injected into the blood stream it is markedly impeded from entering normal brain by the blood-brain barrier. However, direct intracerebral injection circumvents the blood-brain barrier and allows

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<th>Time</th>
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<tr>
<td></td>
<td>Whole Head</td>
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<td>&quot;O&quot;</td>
<td>710.8</td>
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<td>2 min</td>
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<td>1 hr</td>
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<td>72 hrs</td>
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*Data on intravenous injection obtained from a previous study. Values are mean % MBC.
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the drug to penetrate widely and to persist for long periods (Figs. 1, 5, and 7).

In the subcutaneous and intracerebral tumors, directly injected MTX-3H also showed widespread, rapid dispersion. The pattern of accumulation was similar to that seen after intravenous injection but much more rapid. Initially, the location of MTX-3H after intraneoplastic injection was mainly interstitial (Fig. 4) and at later times the distribution was mainly in neoplastic cells (Fig. 8 left). However, even at 2 minutes after intraneoplastic injection some tumor cells were heavily labeled (Fig. 4). Thus, the speed of cellular uptake was greater after intraneoplastic injection since it was extremely rare to find heavy cellular labeling at this time with the intravenous route of injection.

The hope that intraneoplastic injection would improve the penetration of the drug into tumor deposits relatively inaccessible to systemically administered drug was only partially fulfilled. Even at the later time intervals there was still less drug at the margin of the tumor and intraventricularly than at the center (Fig. 8).

There have been several attempts to treat human brain tumors with direct intraneoplastic injections of MTX, either alone or combined with ventricular perfusion. The present study has provided information that may account for the poor results found clinically in these studies with direct injection of MTX. The scintillation counting studies showed that there was rapid reabsorption of MTX-3H after the intracerebral injections and the autoradiographic studies showed poor penetration of MTX-3H into certain areas of the intracerebral tumors. Both these factors would tend to reduce the effectiveness of MTX. For example, rapid reabsorption into the blood would limit the time the neoplastic cells were exposed to the drug and this would be especially disadvantageous for the chemotherapy of brain tumors with a drug like MTX. Methotrexate is a cycle-specific chemotherapeutic agent that exerts its effect during S-phase, and in many types of human gliomas S-phase is rather prolonged. Any further attempts to use direct injections of MTX for brain-tumor chemotherapy would have to take into consideration these important limitations.

It is known from clinical and experimental studies that there is minimal neurotoxicity from intrathecal or intraneoplastic injection of MTX. However, some complications have been reported and this factor requires much more study before definite conclusions can be made about the neurotoxicity of intraneoplastic injections of this drug in the treatment of patients with brain tumors. The finding of highly labeled neurons and glial cells in some of the animals after intracerebral injection suggests that caution is warranted.

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References


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