The Uptake of Tritiated Methotrexate by an Experimental Glioma

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SUMMARY

The uptake of tritiated methotrexate (MTX-3H) was studied in a s.c.-implanted mouse ependymoblastoma and normal mouse brain after a single i.v. injection. An autoradiographic technique suitable for diffusible tracers was used, and autoradiographs of tumor and brain were prepared 2, 10, and 60 min after injection.

At 2 min, the tumors showed a large amount of MTX-3H in the interstitial space and much less in tumor cells. At 10 min, the high interstitial uptake persisted, but in addition most neoplastic cells had become labeled. The tumor showed very little remaining interstitial radioactivity but very high cellular uptake 60 min after injection. In the brain at all times after injection, there was very little extravascular uptake.

These studies show that this glial tumor rapidly accumulated MTX-3H from the bloodstream. Initially, interstitial uptake was high, but cellular uptake was delayed. However, once cellular uptake occurred, it persisted for at least 60 min.

Further studies are needed for determination of how long MTX-3H remains in the neoplastic cells and for comparison of uptake in intracerebral and s.c. implants of the ependymoblastoma.

INTRODUCTION

Although many chemotherapeutic agents have been used in patients with malignant gliomas, beneficial results have been rare (19, 29). There are many possible reasons for this failure. Parenterally or p.o. administered chemotherapeutic agents may fail to reach intracerebral growths in sufficient concentration (28). Agents taken up by brain tumors after p.o., parenteral, or local administration may fail to penetrate into neoplastic glial cells, or agents taken into the cells may fail to exert a chemotherapeutic effect. Unfortunately, on the basis of presently available data, none of these possibilities can be excluded. Indeed, there have been very few studies on the rate of uptake, amount of uptake, and sites of distribution of chemotherapeutic agents in malignant brain tumors. For example, intracellular uptake of these drugs by the neoplastic cells of malignant gliomas has never been demonstrated, although gross uptake into glioma tissue has been shown with MTX2 (9) and uptake into the ether-soluble fraction of gliomas has been shown with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (10). A knowledge of the uptake and distribution of chemotherapeutic agents in malignant gliomas might explain their therapeutic shortcomings and lead to measures which might improve their clinical usefulness. In this paper, the ability of MTX-3H to penetrate into interstitial and intracellular sites in a s.c.-implanted malignant mouse glioma and in normal mouse brain is examined. MTX was chosen, as it has been one of the most commonly used agents for brain tumor chemotherapy (12, 13, 15, 17, 18).

MATERIALS AND METHODS

Tumors. A mouse ependymoblastoma obtained in 1963 from Dr. L. C. Scheinberg of the Albert Einstein College of Medicine, Bronx, N.Y., and maintained in our laboratory by serial s.c. transplantation every 2 weeks during the past 8 years was used. The tumor was originally induced by Zimmerman and Arnold (31) in mouse brain with intracerebrally implanted methylcholanthrene.

Animals. C57BL/6J female mice weighing 16 to 18 g were obtained from Jackson Memorial Laboratory, Bar Harbor, Maine, and were given a s.c. injection in the right lower abdominal quadrant of 0.1 ml of a suspension of tumor which had been ground in 0.9% NaCl solution with mortar and pestle to a consistency which would pass through a 20-gauge needle. The animals were used for injection of MTX-3H 2 weeks after tumor transplantation.

Tracer. MTX-3H was obtained as nominally labeled MTX-31,51,2H from Amersham/Searle Corp., Toronto, Ontario, Canada. Two 1.0-mCi batches were obtained at specific activities of 5.2 and 6.3 Ci/mmole, respectively. Radiochemical purity was assessed by paper chromatography by the suppliers in 2 systems, 0.5% sodium carbonate and 1-butanol:pyridine:water (1:1:1), and was >96% and 98%, and 97% and >98% in the 2 systems for the 2 batches. Each batch was stored for 2 days at −15°C; dissolved in 2.0 ml of a NaOH-NaCl mixture, pH 8.4; and diluted with 2.0 ml of 0.9% NaCl solution.

Injection of Tracer. Six tumor-bearing mice received an i.v. injection of the diluted MTX-3H solution through a tail vein. The volume injected was on the basis of 0.3 ml containing 75 μCi of MTX-3H per 20 g of mouse. For the 2 batches, this dose delivered 0.0054 or 0.0065 mg of MTX per 20 g of mouse. These doses would be comparable to doses of 18.9 or 22.8 mg/70 kg of man.

Sampling Technique. Two mice were sacrificed at each time interval.
interval 2, 10, and 60 min after injection. Under light ether anesthesia, 2-cu mm samples of tumor were excised, placed on minced muscle or liver, mounted on screws, and immediately plunged into 2-methylbutane cooled in liquid nitrogen. Approximately 15 sec elapsed between tumor excision and immersion in 2-methylbutane. The animals were then rapidly decapitated, the skull was opened, and 2-cu mm samples of brain were excised from the right parietal region. The brain samples were then treated in the same way as the tumor samples.

**Autoradiography.** Autoradiographs of tumor and brain were prepared by the technique of Stumpf and Roth (20–23). The frozen tissues were sectioned at 2μ in a Harris cryostat, Model MS-100A, at −30 to −50° with an International Equipment Company Minot ultrathin sectioning microtome, Model 3311. Sections were dried overnight by cryosorption pumping in a Delmar Stumpf-Roth cryosorption pump Model 7127 containing Linde molecular sieve type 5A. The dried sections were then mounted dry in the darkroom on microscope slides previously coated with Kodak NTB3 emulsion diluted 1:1 with distilled water. Exposure was at −15° in the presence of anhydrous calcium sulfate for 20 to 80 days. The autoradiographs were developed in Kodak D-72 for 1.5 min at 19°, fixed in Kodak rapid fixer with hardener, and stained with methyl green pyronin Y. Autoradiographs of tumor and brain from animals that did not receive MTX-3H were prepared in a similar fashion to act as controls.

**RESULTS**

Uptake and Distribution of MTX-3H in Ependymoblastoma. Within 2 min of i.v. injection of MTX-3H, a large amount of the drug had already entered the tumor (Fig. 1), indicating rapid passage across the capillary walls. The drug showed a specific pattern of distribution at 2 min, being mainly in the interstitial space (Fig. 2) with a much smaller number of grains overlying the neoplastic cells. Some neoplastic cells had no overlying grains at this time, and others were only lightly labeled. At 10 min, there was still a large number of grains over interstitial fluid sites, but most cells had become labeled (Figs. 3 and 4). At 60 min, very little remained in the interstitial space, and almost all the drug in the tumor at this time was in the neoplastic cells (Figs. 5 and 6). Although all the neoplastic cells were heavily labeled at 60 min, the distribution of the drug varied, with some cells showing predominantly nuclear uptake and others showing a mixture of nuclear and cytoplasmic uptake (Figs. 6 and 7). The amount of radioactivity in the bloodstream in the tumors at 60 min (Fig. 7) was much less than at the earlier time intervals. The total amount of extravascular radioactivity in the tumors at 60 min appeared less than at the earlier times. Necrotic areas of the tumors contained much less radioactivity than viable areas at all times studied.

Uptake and Distribution of MTX-3H in Normal Brain. At all times after injection, almost all the grains in the autoradiographs of normal brain appeared to be over the lumina of blood vessels (Fig. 8), and there was a progressive decrease in the number of grains with time.

**Controls.** Autoradiographs of tumor and brain from animals which did not receive injections of radioactive tracers showed no greater number of grains over the tissues when compared with the background grain count, which averaged 1 grain/500 sq μ.

**DISCUSSION**

The purpose of this investigation was to evaluate the speed of uptake, distribution, and duration of retention of a chemotherapeutic agent in a malignant glioma. To my knowledge, this is the first autoradiographic demonstration of the uptake of a chemotherapeutic agent in a neoplasm. A glial tumor was chosen because the ultimate aim of these investigations is to explore the reasons for the failure of chemotherapeutic agents significantly to benefit patients with malignant gliomas.

Autoradiography with MTX-3H would seem to be an excellent technique for studying the uptake and distribution of a chemotherapeutic agent in gliomas and other tumors because of the known in vivo stability of MTX and MTX-3H. In all species studied, there is virtually no in vivo metabolism of MTX (3, 5). Studies with MTX-3H prepared by catalytic exchange labeling of MTX (6–8) or by halogen-tritium exchange reaction from the corresponding dichloro compound (1, 32) have shown that the label is stable in vivo and that there is almost complete recovery in the urine and feces of the intact labeled molecule. There is also some evidence that the labeled molecule is stable in neoplastic tissues and that tumors metabolize little, if any, MTX-3H. For example, the distribution and excretion of MTX-3H in tumor-bearing animals paralleled that found in normal animals (6), and patients with malignant neoplasms excreted more than 90% of the unchanged drug in the urine in the first 24 hr (1). Therefore, it is likely that most, if not all, the radioactivity detected in tumor and brain in the present studies represents intact molecules of MTX-3H.

Autoradiographic studies of drug distribution must take into account the diffusibility of the drug during processing. When the degree of tissue binding is uncertain or incomplete, autoradiographic techniques which prevent translocation of the compound must be used. The present technique results in the least possible diffusion during histological and autoradiographic preparation (20–23).

The very rapid passage of MTX-3H across the vascular wall into the interstitial space of the tumor was impressive and indicates that this water-soluble, ionized compound is capable of penetrating into a tumor of glial origin. However, this tumor was growing s.c. and not in the brain. The exceedingly low uptake in normal brain conforms with the known relative inability of compounds of this nature to pass across the blood-brain barrier in normal brain (16, 24). In the tumor, there was a definite time lag between interstitial and intracellular uptake. There was much less labeling of neoplastic cells at 2 min (Fig. 2) than at the later time intervals. At 10 min, most of the cells were lightly labeled (Fig. 4), but at 60 min all were heavily labeled (Figs. 6 and 7). The reason for this delay in intracellular uptake is not known but may be related to the retention in this glial tumor of some of the characteristics of normal brain, such as the lower uptake of
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water-soluble compounds. The uptake of radioiodinated human serum albumin into the interstitial fluid and neoplastic cells of the mouse ependymoblastoma was even more delayed, as shown by conventional autoradiography (25). However, with tritiated hydrocortisone in the same tumor (26) there was virtually no delay, and high intracellular uptake was found 2 min after i.v. injection. The latter study used the same autoradiographic technique as the present study with MTX-3H.

The disappearance of MTX-3H from the interstitial space in the 60-min specimens probably reflects the rapid decline in blood levels of the drug. The biological half-life of MTX-3H in blood in dogs and monkeys was 75 to 90 min (6). The retention of the tracer in the cells in the 60-min specimens must indicate that once the tracer enters neoplastic cells it attaches itself to some cell component strongly enough to resist being drawn back into the interstitial fluid and bloodstream.

Other studies have suggested cellular uptake of MTX. Kinetic studies in mouse leukemia (4) and in a bacterium (30) have shown results suggestive of an active transport mechanism. In rat liver homogenates, Werkheiser (27) has shown increased activity of the enzyme dihydrofolate reductase and binding of MTX to the enzyme, and Henderson et al. (6) showed that in mouse liver most of the MTX-3H was in the 70,000 X g supernatant, which is consistent with binding to dihydrofolate reductase, which resides in the soluble fraction. This study provides the strongest evidence for intracellular uptake in neoplasms.

The intracellular sites of uptake of MTX-3H were variable, with some cells showing predominantly nuclear labeling and others showing a mixture of nuclear and cytoplasmic labeling. MTX is a folate acid antagonist and therefore is considered to be one of the cycle-specific chemotherapeutic agents (2). It may be that the distribution of intracellular labeling is related to the phase that a cell was in when MTX-3H entered the cell.

Autoradiography has an important role to play in the study of the distribution of drugs within brain tumors. Zonal centrifugation (11, 14) and physiological “space” studies also provide information on distribution, although the results of the latter must be interpreted with caution. For example, Levin et al. (9) found that in s.c. implants of mouse ependymoblastoma the MTX-3H space averaged 47% as compared with an inulin space of 39%, which would indicate uptake in a space only slightly larger than the extracellular space. Their determinations were done under steady state conditions in nephrectomized animals and with an “injection schedule to maintain relatively constant plasma levels of MTX.” It is uncertain what the autoradiographic distribution of MTX-3H would be under these conditions. On the other hand, if one had space studies for determining distribution, it is doubtful that the early interstitial and later intracellular localization of MTX-3H could be detected.

Further information on the uptake of chemotherapeutic agents is required to achieve our aim of improving the clinical usefulness of these agents in patients with malignant brain tumors. One obvious necessity is to determine what differences there may be between uptake in s.c. versus intracerebral implants of the mouse ependymoblastoma. It has been shown by Levin et al. (9) that the MTX-3H space averages 153% higher in s.c. than intracerebral implants of this tumor. The present experiments were terminated 60 min after i.v. injection and showed persisting high cellular labeling. Studies at later time intervals should be done to see how long cellular labeling persists after a single i.v. dose. This may provide a rational basis for selection of dose frequency with drugs such as MTX, a problem not yet solved (2). Studies to provide some of these answers are now in progress.

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REFERENCES


Figs. 1 to 8. Autoradiographs of mouse ependymoblastoma and brain: 2-μ-thick sections; exposure time, 20 days; methyl green pyronin Y stain. Fig. 1. Ependymoblastoma 2 min after injection of MTX-3H. The grains are principally over the interstitial space. X 660.

Fig. 2. Ependymoblastoma 2 min after injection of MTX-3H. The clear areas representing interstitial space contain most of the grains, while the neoplastic cells contain few. X 1700.

Fig. 3. Ependymoblastoma 10 min after injection of MTX-3H. There is now an even distribution of grains between interstitial space and intracellular sites. X 660.

Fig. 4. Ependymoblastoma 10 min after injection of MTX-3H. As compared with Fig. 2, many more grains overlie the neoplastic cells, although an equally large number still lie over the interstitial space. X 1700.

Fig. 5. Ependymoblastoma 60 min after injection of MTX-3H. The clear areas representing interstitial space are now almost devoid of grains. X 660.

Fig. 6. Ependymoblastoma 60 min after injection of MTX-3H. Most of the grains overlie neoplastic cells with very few remaining over the interstitial space. X 1700.

Fig. 7. Ependymoblastoma 60 min after injection of MTX-3H. A large blood vessel with grains over its lumen is seen in the center of the photomicrograph. Most of the grains are over the neoplastic cells. X 1700.

Fig. 8. Normal brain 10 min after injection of MTX-3H. Almost all the grains are present over the lumen of the blood vessel in the center of the photomicrograph. X 1700.
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