Uptake of tritiated methotrexate by mouse brain tumors after intravenous or intrathecal administration

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Malignant gliomas were induced in strain ddN mice by intracerebral implantation of a 20-methylcholanthrene pellet. The uptake and distribution of tritiated methotrexate (MTX-3H) in the tumor were investigated by radioactive assay and radioautography after single intravenous or intrathecal injections. By either route, a large amount of MTX-3H was taken up by gliomas, and a significantly higher concentration was observed in tumor than in the brain tissue. At 24 hours after intrathecal administration, the uptake of MTX-3H by gliomas exceeded that achieved after intravenous injection, although the drug dosage in the latter was 10 times that in the former.

KEY WORDS • brain tumor • chemotherapy • drug distribution • methotrexate • blood-brain barrier • cerebrospinal fluid

CHEMOTHERAPY is a promising treatment for patients with malignant glioma, although it has not yet been proved sufficiently effective. In this form of therapy an adequate uptake of the effective drug by the tumor is of fundamental importance. The present study was designed to investigate the uptake of a cytostatic agent by mouse glioma and to compare the intravenous and intrathecal administration of this drug.

Materials and Methods

Induction of Tumors

Brain tumors were induced by the technique of Zimmerman and Arnold in 5-week-old male mice of strain ddN, weighing approximately 15 gm. The animals were anesthetized with ether, a small craniotomy was made in the right parietal region, and a pellet of 20-methylcholanthrene approximately 1 mg in weight was implanted intracerebrally under sterile conditions using a surgical microscope. The bone flap was replaced and fixed with a surgical adhesive.* The animals were carefully observed, and when malaise, neurological signs, or bulging of the skull was noted, tritiated methotrexate was injected intravenously or intrathecally.

We used tritiated methotrexate (methotrexate-3', 5'-3H, sodium salt: MTX-3H)†

*Surgical adhesive manufactured by Aron-Alpha, Sankyo, Japan.
†Tritiated methotrexate manufactured by the Radiochemical Center, Amersham, Buckinghamshire, England.
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with specific activities of 0.55, 44, 29, and 23 mCi/mg. The radiochemical purity was assayed by paper chromatography by the suppliers in two systems, 0.5% sodium carbonate, and n-butanol:pyridine:water (1:1:1), and was greater than 98% in the two systems for each batch. During the experimental period, the stock compound was frequently rechromatographed in the above systems, and the radiochemical purity of MTX-\(^3\)H was verified in excess of 96%. The MTX-\(^3\)H with specific activity of 0.55 mCi/mg was prepared by addition of cold methotrexate* and administered to the symptomatic mice intravenously in a single dose of 1.8 \(\mu\)g/gm body weight through a tail vein or intrathecally in a single dose of 0.18 \(\mu\)g/gm body weight by the technique of Lindberg and Ernster.\(^{15}\)

Radioactive Assay

The mice that had received MTX-\(^3\)H were bled to death by cardiac puncture at varying times after injection. Immediately after death, samples of tumor, normal brain tissue, liver and kidney were removed, stored in closed vials and weighed accurately as soon as possible in a cold room, while a piece of the induced tumor was examined histopathologically. The radioactive of these tissues was assayed by the method of Dulcino, et al.,\(^4\) and the radioactivity of plasma assayed by the method of Meade and Stiglitz.\(^{16}\) The scintillating mixture was stored for 24 hours, and the counts were made with the liquid scintillation counter.\(^4\) The counting efficiency was 20% to 40%. The final data of the tissue radioassay were expressed as disintegration per min (dpm) per gm (ml) of wet tissue (plasma).

Radioautography

Light microscopic radioautographs were prepared by a method for diffusible molecules described by Wilske and Ross.\(^{28}\) The radioactive drug injected was in the same dose of MTX-\(^3\)H used in the above experiment but with higher radioactivity (23 or 44 mCi/mg, radiochemical purity > 98%). The animals were sacrificed by exsanguination, some at 1 and some at 24 hours after injection of MTX-\(^3\)H. The organs and tissues were immediately removed and cut into 1 mm cubes with sharp razor blades under a dissecting microscope in a cold room. The tissue blocks were quickly frozen in isopentane suspended in a bath of liquid nitrogen. Frozen tissue blocks were then placed in a freeze-dry apparatus‡ at \(-40^\circ\) C, 10\(^{-3}\) atmospheric pressure for 96 hours. After drying was completed, tissues were fixed in the vapor phase of paraformaldehyde at 60\(^\circ\) C for 24 hours and embedded in Epon. Sections were made 1½ \(\mu\) thick and coated with liquid emulsion§ by dipping. Following exposure for 4 to 7 weeks, the sections were developed, fixed, washed, and stained with Azur II-methylene blue.

Results

Induced Tumors

One hundred symptomatic mice each received a single injection of MTX-\(^3\)H, 46 intravenously and 54 intrathecally. The tumor was found to be successfully induced in 83% of these animals; 57 tumors were intracranial and 32 extracranial. The histopathological types and average induction time (the period from implantation of carcinogen to sacrifice) are listed in Table 1.

Of the intracranial tumors, 41 were malignant gliomas histologically identical to a human malignant mixed glioma or glioblastoma (Fig. 1); six were fibrosarcomas and one was a squamous cell carcinoma. The remaining nine were extracerebral fibrosarcomas probably arising from meninges. In six mice tumors were induced both intracranially and extracranially.

Radioactive Assay

The data obtained from animals that had

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* Methotrexate manufactured by Lederle Laboratories, Pearl River, New York 10965.
† Liquid scintillation counter Mark I manufactured by Nuclear Chicago, 2000 Nuclear Drive, Des Plaines, Illinois 60018.
‡ Freeze-dry apparatus Daia type manufactured by Daia Vacuum Engineering Co. Ltd., 7-3-7 Minamisuna, Kotoku, Tokyo, Japan.
§ Liquid emulsion NR-M2 manufactured by Konishiroku Photo Industry Co. Ltd., 3-1 Muro-machi, Nihonbashi, Tokyo, Japan.
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TABLE 1

Types of tumor induced in mice

<table>
<thead>
<tr>
<th>Type of Tumor</th>
<th>No. of Mice</th>
<th>Average Induction Time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>intracranial tumors:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>malignant mixed glioma</td>
<td>41</td>
<td>268</td>
</tr>
<tr>
<td>sarcoma in the brain</td>
<td>6</td>
<td>198</td>
</tr>
<tr>
<td>squamous cell carcinoma in the brain</td>
<td>1</td>
<td>178</td>
</tr>
<tr>
<td>meningeal sarcoma</td>
<td>9</td>
<td>215</td>
</tr>
<tr>
<td>total</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>extracranial tumors:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sarcoma of the scalp</td>
<td>31</td>
<td>191</td>
</tr>
<tr>
<td>squamous cell carcinoma</td>
<td>1</td>
<td>107</td>
</tr>
<tr>
<td>total</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>no tumor</td>
<td>17</td>
<td>268</td>
</tr>
<tr>
<td>total</td>
<td>106*</td>
<td></td>
</tr>
</tbody>
</table>

*Double tumors (intracranial and extracranial) were induced in 6 mice.

received a faulty injection of MTX-3H or had died before sacrifice were excluded from the results. Successful radioactive assays were performed on 31 intracranial intracerebral tumors (27 gliomas, 3 sarcomas, and 1 carcinoma), seven intracranial extracerebral (meningeal) sarcomas, and 28 extracranial tumors.

Large amounts of MTX-3H were found in gliomas after either intravenous or intrathecal administration (Fig. 2); there was, however, some variation particularly in determinations made soon after administration of the drug. The relatively high drug levels in the gliomas were maintained for more than 24 hours.

In all cases, the MTX-3H levels in gliomas after either intravenous or intrathecal administration, and the average concentration ratios of tumor to brain were 3.41 ± 2.55 and 4.77 ± 3.35 after intravenous and intrathecal administration respectively. Comparison of the drug concentrations in tumor and brain tissue, and of the tumor/brain concentration ratios for the different types of tumor and the different administration routes of the drug yielded the data in Table 2.

In gliomas, the tumor MTX-3H level 24 hours after intrathecal administration ranged from $6.94 \times 10^4$ dpm/gm to $21.20 \times 10^4$ dpm/gm, with an average of $15.18 \pm 5.38 \times 10^4$ dpm/gm. This exceeded that achieved after intravenous administration, which ranged from $4.21 \times 10^4$ dpm/gm to $13.30 \times 10^4$ dpm/gm, with an average of $9.81 \pm 3.62 \times 10^4$ dpm/gm, although the dosage of the latter was tenfold greater than that of the former.

There was no apparent difference in the MTX-3H level of gliomas and other intracerebral tumors after either intravenous or intrathecal administration. With meningeal sarcomas, the tumor drug levels after intrathecal administration (4.62 ± 2.44 x 10^4 dpm/gm) were approximately equal to those after intravenous administration (6.27 ± 1.41 x 10^4 dpm/gm), but were significantly lower than those of glioma after intrathecal administration (15.18 ± 5.38 x 10^4 dpm/gm) (p < 0.01).

No significant difference was found in the tumor concentration of MTX-3H in extracranial scalp sarcomas and intracerebral tumors after intravenous administration. Table 3 summarizes the data obtained from four mice with double tumors separately induced intracranially and extracranially. In mice, there is apparently a higher MTX-3H level for gliomas than for scalp sarcomas after intrathecal administration.

The concentration of MTX-3H in the brain tissue around the implanted pellet was compared to that of distant brain tissue in mice in which tumor induction failed. There was no significant difference in the two recordings, nor between intravenous and intrathecal administration. The ratios of the concentration in the brain tissue around the pellet to that in the distant brain tissue were 1.11 ± 0.44 for intravenous and 0.96 ± 0.57 for intrathecal administration.

Plasma concentration of MTX-3H after intrathecal administration is apparently lower than that after intravenous administration, particularly soon after administration of the drug. The concentration in the liver and the kidney almost paralleled that in plasma.

Radioautography

Radioautography at 1 or 24 hours after intravenous or intrathecal administration of MTX-3H was successfully performed in 10 mice. At 1 hour after intrathecal
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administration, a moderately large amount of silver grains appeared over the glioma tissue, not only in the peripheral part of the tumor adjacent to the cerebrospinal fluid space, but also in the central part of the tumor remote from that space. The distribution of the grains was variable; in some the grains were concentrated over interstitial space, and in others they were concentrated over glioma cells. However, normal brain tissue, even in the part adjacent to the cerebrospinal fluid space, contained only a small amount of radioactivity; particularly, cellular labeling was seldom seen over either the neuronal or glial cells (Figs. 3 and 4).

The radioautographs of glioma after intravenous administration of MTX-\(^{3}H\) also showed that a large amount of MTX-\(^{3}H\) entered tumor tissue, but there was no remarkable difference attributable to intravenous versus intrathecal administration. Glioma tissue still contained considerable radioactivity 24 hours after intrathecal administration, less interstitially and more intracellularly in the glioma cells. However, in the labeled cells, there was no specially localized distribution of the grains; in some parts, the grains were distributed heavily

Fig. 1. Malignant mixed glioma induced in the mouse brain. Upper Left: Photograph of the brain with an infiltrative tumor in the right hemisphere. Lower Left: Photomicrograph of a portion of anaplastic astrocytoma, showing pleomorphic tumor cells with round or kidney-shaped nuclei in fine network of fibrils. H & E, \(\times 400\). Lower Right: Photomicrograph of a portion of oligodendroglioma, showing dark, bare nuclei surrounded by halos. H & E, \(\times 400\).
over nuclei and in others, over cytoplasm (Figs. 5 and 6).

Radioautographs of the liver and kidney (Fig. 7) confirmed the findings reported by Darzynkiewicz, et al.8 In the liver, silver grains were distributed over parenchymal cells, and in the kidney they were located almost exclusively in the epithelial cells of the proximal convoluted tubules while glomeruli and distal convoluted tubules showed low radioactivity.

Discussion

There has been a considerable amount of research on the uptake of chemotherapeutic agents in human malignant brain tumors after systemic administration. Simon, et al.,24 demonstrated that tritiated cyclophosphamide could penetrate into the various human brain tumors including glioblastoma after intravenous administration; however, no selective concentration of the drug in the tumor was noted. Ojima, et al.,20 reported that intravenous methotrexate entered into a human astrocytoma and a metastatic tumor. Radiometric and radioautographic distribution studies of SP-I-3H and SP-I-14C were performed in human glioblastomas by Meier-Ruge, et al.,17 who found the greatest concentration and slowest rate of disappearance of SP-I in the periphery of the tumor as compared with the center and the normal neighboring brain tissue. Graul, et al.,5 have obtained the same result in their clinical study using tritiated cyclophosphamide and assumed that the drug concentration in the tumor largely depended on the blood supply of the tissue.

Basic studies on the uptake and distribu-

![Table 2](image)

**Figure 2.** Methotrexate-3H (MTX-3H) levels in glioma, brain, and plasma following intravenous (left) and intrathecal (right) injection in mice.

**Table 2**

*Distribution of radioactivity 24 hours after intravenous or intrathecal injection of methotrexate-3H*

<table>
<thead>
<tr>
<th>Type of Tumor</th>
<th>Route*</th>
<th>No. of Mice</th>
<th>MTX-3H Concentration (×10^6 dpm/gm Wet Tissue)</th>
<th>Tumor/Brain Conc. Ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tumor</td>
<td>Brain</td>
</tr>
<tr>
<td>malignant glioma</td>
<td>i.v.</td>
<td>4</td>
<td>9.81 ± 3.62</td>
<td>4.23 ± 1.63</td>
</tr>
<tr>
<td>meningeal sarcoma</td>
<td>i.v.</td>
<td>4</td>
<td>15.18 ± 5.38</td>
<td>2.83 ± 1.31</td>
</tr>
<tr>
<td>sarcoma of scalp</td>
<td>i.v.</td>
<td>6</td>
<td>10.23 ± 2.41</td>
<td>2.68 ± 0.56</td>
</tr>
</tbody>
</table>

*†: p = t test, paired sample; n.s. = not significant.

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**TABLE 3**

*Distribution of radioactivity in double tumor (intracranial and extracranial) in four mice 24 hours after injection of methotrexate-3H*

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Route*</th>
<th>Interval After Admin.</th>
<th>Type of Tumor</th>
<th>MTX-3H Concentration (X 10^4 dpm/gm wet tissue)†</th>
<th>Tumor/Brain Concent. Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>3471</td>
<td>i.v.</td>
<td>6 hr</td>
<td>sarcoma (scalp)</td>
<td>5.31</td>
<td>2.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sarcoma (cerebral)</td>
<td>5.40</td>
<td></td>
</tr>
<tr>
<td>2671</td>
<td>i.v.</td>
<td>14 hr</td>
<td>sarcoma (scalp)</td>
<td>9.01</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sarcoma (cerebral)</td>
<td>11.85</td>
<td></td>
</tr>
<tr>
<td>4871</td>
<td>i.v.</td>
<td>35 min</td>
<td>sarcoma (scalp)</td>
<td>13.63</td>
<td>2.38</td>
</tr>
<tr>
<td>2071</td>
<td>i.t.</td>
<td>24 hr</td>
<td>glioma sarcoma (scalp)</td>
<td>18.04</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>glioma</td>
<td>14.12</td>
<td></td>
</tr>
</tbody>
</table>

*i.v. = intravenous administration, MTX-3H dose: 180 μg MTX (1.0 μCi)/gm; i.t. = intrathecal administration, MTX-3H dose: 0.18 μg MTX (0.1 μCi)/gm.
†10^4 dpm/gm of MTX-3H = 0.008 μg of MTX.

...intravenous administration, MTX readily permeated the extracellular space of murine ependymoblastoma implanted in the cerebrum; the tumor concentration was approximately three times that of adjacent brain tissue and 10 times that of distant normal brain tissue. Recently, Tator also investigated radioautographically the distribution of intravenous MTX-3H in intracerebrally transplanted ependymoblastomas of mice. He demonstrated that in tumor 2 minutes after intravenous administration, the drug was mainly intravascular or interstitial, while at 60 minutes it was mainly intracellular; moreover, the neoplastic cells in the central mass of the tumor were more heavily labeled than those in the periphery.

Intrathecal chemotherapy has been of increasing interest and applied to the treatment of brain tumors including gliomas, several experimental studies on the effectiveness and usefulness of this therapy have been reported.

Several studies have been made of the concentration of various drugs in the cerebrospinal fluid after intrathecal administration. However, there is still little direct information as to the uptake and distribution of cytostatic agents in brain tumor after intrathecal administration, so far as we know. Our present work represents the first systematic comparative study of intravenous and intrathecal chemotherapy with reference to the drug distribution in brain tumor. The use of transplanted brain tumors as an experimental model is subject to criticism particularly in the study on the uptake of drug by tumor, for it is reasonable to presume that the transplanted brain tumor differs from the human primary brain tumor both in its growth characteristics and in the significance of factors having to do with blood-brain barrier. In our study, experimental brain tumors were induced by implanting 20 methylcholanthrene pellets in the cerebrum of mice. The tumors were found to be very similar macroscopically as...
well as microscopically to human brain tumors. The influence of the pellet or its surgical implantation in the brain upon the results of the experiment was almost negligible; the data obtained from the specimens of brain tissue around the pellet in mice in which tumor induction failed showed no significant difference from those of the brain tissue distant to the pellet.

The comparative study was based mainly on data obtained 24 hours after administration of MTX because it is well known that MTX binds firmly to folate reductase and remains in tissue for long periods, and that almost no free MTX is present in the tissue 24 hours after administration.3,8,9,12,26

Our observations demonstrate that the glioma takes up both venous and intrathecal

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**Fig. 3.** Malignant mixed glioma. *Left:* Coronal section of the brain, showing a pellet surrounded by the induced tumor. *Right:* Drawing of the brain tumor, illustrating the sites of radioautographs shown in Fig. 4.

**Fig. 4.** Radioautographs of the glioma (*left*) and brain (*right*) 1 hour after intrathecal injection of 50 μCi of MTX-3H. *Left:* Site A shown in Fig. 3. Silver grains are numerous in the malignant mixed glioma some distance from the subarachnoid space. *Right:* Site B in Fig. 3. Silver grains are rare in the subpial region of the brain adjacent to the tumor. 1.5 μ-thick sections; exposure time, 7 weeks; Azur II-methylene blue stain, × 1000.
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MTX in considerably larger amounts and in a significantly greater concentration than does brain tissue. The variation in animals may have been related to differences in the size, site, and character of induced tumors, particularly in the case of intrathecal administration, as dictated by differences in the state of the cerebrospinal fluid space in each animal.

Intravenously administered MTX-3H entered selectively into glioma tissue of the brain, and there was no significant difference between the amount of MTX-3H taken up by intracerebral tumors including gliomas and the uptake by extracranial scalp sarcomas. The evidence indicates that there may be a breakdown or lack of blood-brain barrier in the brain tumor tissue, a result we had anticipated because of the nature of clinical information derived from brain scans in patients with brain tumor.

It is interesting that intrathecal drug administration achieved selective distribution of the drug in brain tumor tissue. The exact mechanism is not yet fully understood but possible explanations are: 1) most of the induced tumors in our study were adjacent to the cerebrospinal fluid space; 2) the extracellular space of tumor tissue is much greater than that of normal brain tissue; 3) the rate of diffusion of the drug from cerebrospinal fluid tissue into tumor tissue may exceed that into normal brain tissue; 4) there may be a difference in the distribution of folate reductase to which MTX would bind in the tissue. These factors, combined with our demonstration that the amount of MTX tumor uptake 24 hours after intrathecal administration exceeded that after a much higher intravenous dosage, are highly suggestive of the ultimate effectiveness and usefulness of intrathecal administration of cytostatic agents in the clinical chemotherapy of brain tumors.

The radioautographic evidence obtained
in this study indicates that both intravenously and intrathecally administered MTX-3H enter intracellularly into the neoplastic cells of gliomas. However, we did not obtain characteristic distributions of MTX-3H in the tumor from the radioautographs 1 and 24 hours after drug administration in the present study. The earlier examinations after drug administration may prove more suitable for this purpose.

We believe the most important observation derived from this study is that intrathecal chemotherapy may be more effective than intravenous chemotherapy in the treatment of a malignant brain tumor adjacent to the cerebrospinal fluid, although both routes may be useful.

**Summary**

The uptake and distribution of tritiated methotrexate (MTX-3H) in brain tumors induced by intracerebral implantation of a 20-methylcholanthrene pellet in mice were investigated by radioactive assay and radioautography. The following evidence substantiates the usefulness of methotrexate chemotherapy in the treatment of brain tumor, and emphasizes the advantage of intrathecal administration of the drug when the tumor is adjacent to a cerebrospinal fluid space.

1. A considerable amount of MTX-3H was selectively distributed in gliomas of the brain after either intravenous or intrathecal administration.
2. There were significant differences between the concentration of MTX-3H in tumor and brain tissue after either intravenous or intrathecal administration.
3. The amount of MTX-3H in glioma tissue at 24 hours after intrathecal administration exceeded that after intravenous administration, although the drug dose of the latter was tenfold greater than that of the former.
4. There was no significant difference between the concentration in glioma tissue and that in extracranial scalp sarcoma tissue.
5. Radioautographic study revealed that
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MTX-3H was distributed selectively in glioma tissue of the brain, penetrated into the neoplastic cells, and remained intracellularly for more than 24 hours after either route of administration.

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References


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