Methotrexate Neurotoxicity: In Vitro Studies Using Cerebellar Explants from Rats

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ABSTRACT

The mechanism of methotrexate (MTX)-induced neurotoxicity was investigated using cerebellar explant cultures from fetal rats. After 3 weeks of growth, myelinated cultures were treated with MTX at 1 μM, lyssolecithin at 1 mg/dl, or unaltered nutrient medium. Myelin sheaths devoid of axons were observed by histological and electron microscopic preparations after 2 weeks of MTX exposure. After 5 weeks, cultures were almost entirely devoid of myelin sheaths. Myelin basic protein in the media removed from the cultures showed an increase in concentration after 3 weeks of MTX exposure and was significantly greater than control after 5 weeks of exposure. 2',3'-Cyclic nucleotide 3'-phosphohydrolase activity, a measure of oligodendroglial function, was not significantly different in the MTX group compared to controls. Lyssolecithin-treated cultures showed widespread destruction and an early increase in myelin basic protein release into the medium. These data indicate that, in the cerebellar explant cultures, MTX is primarily a neuronal toxin, and the demyelination is a consequence of axonal loss and is not related to a change in oligodendroglial cell function. These findings provide new insight into the pathogenesis of MTX-induced neurotoxicity.

INTRODUCTION

Systemic or i.t. administration of MTX has been shown to produce a chronic progressive neurotoxic syndrome known as leukoencephalopathy (1–3). Patients with this disorder usually present with personality changes which may slowly progress to frank dementia, coma, or death. Less commonly, ataxia, seizures, and focal neurological signs such as hemiparesis or bulbar palsy may occur (2, 4, 10, 12). These symptoms usually develop months to years after MTX treatment and are most common in patients who receive cranial irradiation prior to MTX (4, 7, 9). Histologically, the most striking abnormalities are found in the subcortical white matter of the cerebral hemispheres and cerebellum. Coagulative necrosis of white matter is usually surrounded by areas of axonal swelling and disruption, demyelination, and reactive astrocytosis with a relative absence of an inflammatory response (4, 11). The pathogenesis of MTX-related leukoencephalopathy remains unknown. Pathological studies have been limited to patients with profound toxicity and often reveal only widespread necrosis. Attempts at developing animal models of MTX-induced leukoencephalopathy have not successfully reproduced the delayed demyelination observed clinically (6).

This paper describes experiments that utilize cerebellar explant cultures to study MTX neurotoxicity. This organotypic culture system has been widely used to study the neurotoxicity of heavy metals such as methyl mercury and tin (10, 11). In these heavy metal studies, changes in the cultures closely resembled changes seen with human exposure. The explant culture system preserves normal intercellular relationships among neuronal elements, permits normal maturational events such as myelination to occur, and eliminates problems of variable drug delivery to brain tissue. MTX exposure to these explant cultures resulted in primary diffuse axonopathy and a secondary loss of the myelin sheath with preservation of oligodendroglial function. These findings provide new insight into the pathogenesis of MTX-induced neurotoxicity and suggest that the delayed demyelination seen following treatment with MTX results primarily from toxicity to neurons.

MATERIALS AND METHODS

Establishing the Cerebellar Explant Cultures. Cerebellar explant cultures were established using the technique of Yonezawa et al. (12). All experimental techniques involving animals were approved by The Johns Hopkins Medical Institution Animal Care Committee. Pregnant rats at 21 days of gestation (Charles River, Kingston, NY) were deeply anesthetized using methoxyflurane (Metaflane; Pitman-Moore, Washington Crossing, NJ). The uterus was removed under aseptic conditions and placed in HBSS (GIBCO, Grand Island, NY). Each fetus was removed from the amniotic sac under a laminar flow hood. The cerebellum was removed and sagittally sliced into 0.5-mm-thick sections. Two tissue sections were placed on a rat tail collagen-coated coverslip and two drops of nutrient medium were added. The nutrient medium was composed of 40% heat-inactivated defined fetal calf serum (Hyclone, Logan, UT), 25% α-minimal essential medium (GIBCO), 35% HBSS (GIBCO), glucose at 6 mg/ml, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (GIBCO) at 10–4 M, and low zinc insulin at 1 μg/ml (Squibb-Nov sk, Princeton, NJ). The coverslip was then placed inside a Maximow slide assembly (Rochester Scientific, Rochester, NY), sealed with paraffin, and placed in an incubator at 35°C. Twice each week, the old medium was washed from the coverslip using 200 μl HBSS, two drops of fresh medium were added, and the tissue sections were sealed in a new Maximow slide assembly.

Experimental Design. Myelination was assessed by phase contrast microscopy after the cultures were maintained for 3 weeks. Cultures which did not show evidence of myelin on the neurites extending from the explants were discarded. The remaining cultures were randomly divided into three groups with ten Maximow slide assemblies in each group. The nutrient medium used for the first group contained 1 μM MTX. This medium was used twice each week in the routine maintenance of the cultures. The second group of cultures were maintained with nutrient medium containing lyssolecithin (Sigma, St. Louis, MO), 1 mg/dl. The third group continued to receive normal nutrient medium. The experiment was carried out for 5 weeks from the time of randomization of the cultures.

Histological Methods. Cultures were processed for light and electron microscopy at 2 and 5 weeks of exposure. Cultures were first rinsed in cold 0.1 M phosphate buffer, pH 7.4, twice for 5 min each. The tissue was fixed in situ for 1.5–2 h in 2% glutaraldehyde-1% paraformaldehyde-0.1 M phosphate buffer, pH 7.4 at 4°C. The fixative was rinsed off with 0.1 M phosphate buffer, 1% sucrose, pH 7.4 at 4°C, 3 times for 15 min each. Post-fixation was performed for 2 h in 1% OsO4 in 0.1 M phosphate buffer. Routine dehydration was performed through graded ethanol into 100% Polybed/812. Light vacuum conditions were used to enhance resin penetration. The tissue was cured for 48 h in a 60°C oven.

Thick sections (700 nm) were cut with glass knives and stained with
toluidine blue-O/azure II B. Thin sections (60–70 nm) were made with an MJO Diatome diamond knife on an LKB-V ultramicrotome, stained lightly with uranyl acetate and lead citrate, and viewed on a Philips 410 transmission electron microscope.

MBP Analysis. As previously described, medium from each culture was collected twice weekly and stored at −20°C. The MBP content in each sample was measured by radioimmunoassay (Diagnostic Systems Industries, Webster, TX) (13). The medium-HBSS mixture was thawed and 10 µl were removed for total protein determination. The total protein was measured by the Bradford assay (Bio-Rad, Rockville Center, NY) (14). The remainder of the medium sample was placed in a polypropylene tube with 200 µl of anti-MBP antisera. The mixture was incubated at room temperature for 24 h. 125I-MBP was added to each tube and incubated for 4 h at room temperature; precipitating antibody was then added and allowed to incubate for 30 min. One ml of water was mixed in each tube and the suspension was centrifuged at 1500 X g for 15 min. The supernatant fraction was decanted and the pellet was counted for 1 min in a gamma counter. A standard curve was generated with known amounts of MBP and the MBP values (ng/mg protein/ml) of the samples were then calculated using this standard curve.

CNP. Cultures remaining after 5 weeks of exposure were harvested for CNP activity. This was assayed using the method of Prohaska et al. (15). Cultures were washed with HBSS, removed from the collagen-coated coverslip, weighed, and placed in a test tube containing 9 volumes of 3 M sucrose. The tissue was homogenized with a Teflon pestle. Sodium deoxycholate, 1% (w/v), and 0.2 M Tris-HCl at pH 7.5 were added and the tissue was rehomogenized. Ten µl of the homogenate were removed for total protein concentration determination using the Bradford assay. Ten µg of protein from the sample were then combined with 10 mM 2',3'-cyclic AMP-0.1 M Tris-maleate buffer. The mixture was incubated at 30°C for 10 min and placed in boiling water for 30 s to stop the reaction. Escherichia coli alkaline phosphatase was added to cleave the 2'-AMP and was allowed to incubate for 20 min at 30°C. Free P1 was extracted with sec-butyl alcohol-toluene (modification of the cited reference) and measured by UV spectrophotometry (410 nm). One unit of enzyme activity, defined as the amount that produces 1 µmol of 2'-AMP from 2',3'-cyclic AMP per min, was standardized to units/mg total protein.

Statistical Analysis. Statistical comparisons between the MBP and CNP results of each group at each time point were evaluated using analysis of variance with the Scheffe test to allow accurate comparison of time-dependent variables. Statistical comparison of CNP activity data was performed using a two-tailed Student t test. Histological and electron microscopic findings are reported in descriptive form.

RESULTS

Histology. Toluidine blue-stained sections of the three groups of cultures were compared at 2 and 5 weeks of exposure. After 2 weeks of the experiment, cultures treated with MTX showed loss of axons and empty myelin sheaths. The tissue architecture and neuropil remained normal. After 5 weeks of exposure to MTX, myelin was rarely seen in the histological section and mild disruption of the neuropil and scattered pyknotic cells were noted. Lysolecithin-treated cultures showed widespread cellular destruction and no myelin sheaths remained after 2 weeks of exposure. After 5 weeks of exposure to lysolecithin, no viable cells remained. Control cultures maintained normal cytoarchitecture and myelinated axons throughout the course of the experiment.

Electron microscopic analysis of MTX-treated cultures after 2 weeks of exposure confirmed the light microscopic findings. Myelin sheaths devoid of their accompanying axons were noted (Fig. 1a). Normal oligodendroglial processes were seen in association with the retained myelin sheaths. Several myelin sheaths in the MTX-treated specimen showed early signs of degeneration, but many maintained a normal ultrastructural...
appearance. Lysolecithin-treated cultures showed a complete absence of myelin and widespread cellular destruction after 2 weeks of exposure (Fig. 1b). In comparison, control cultures contained many axons with their accompanying myelin sheaths (Fig. 1c).

MBP. Serial measurements of MBP concentration in the media were used as an indicator of demyelination in the cultures. During the first 2 weeks of the experiment, there was no significant difference in MBP concentrations between MTX and control cultures (Fig. 2). However, MBP concentrations in the media rose after 3 weeks of exposure to MTX. By the 5th week of exposure, MBP concentrations in MTX-treated cultures were markedly elevated compared to controls ($P < 0.002$). Lysolecithin-treated cultures had an early increase in MBP concentration compared to controls, consistent with the early widespread destruction seen on histological sections. Control cultures had low concentrations of MBP throughout the course of the experiment.

CNP Activity. CNP activity was measured in the homogenized cultures and in developing rat cerebellum to assess oligodendroglial function. These data demonstrate a developmental increase in CNP activity in vivo during normal myelination that is paralleled during myelination in the culture system (Table 1).

CNP activity in the MTX-treated and control cultures were compared after 5 weeks of the experiment. No significant difference in CNP activity between control and MTX-treated cultures was noted (Table 1). CNP activity was not measured in the lysolecithin-treated cultures because of the absence of viable cells after 5 weeks of lysolecithin treatment.

![Fig. 2. MBP release into the nutrient media, indicating loss of the myelin sheath, over the 5-week course of the experiment. MTX-treated cultures (●), lysolecithin-treated cultures (○), and control cultures (□) were compared.](image)

### Table 1 CNP activity

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>CNP activity (units/mg total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal cerebellum</td>
<td>None detected</td>
</tr>
<tr>
<td>Newborn cerebellum</td>
<td>2.8</td>
</tr>
<tr>
<td>Adult cerebellum</td>
<td>7.9</td>
</tr>
<tr>
<td>Control culture (21 days)</td>
<td>6.0</td>
</tr>
<tr>
<td>Control culture (66 days)</td>
<td>13.4</td>
</tr>
<tr>
<td>MTX-treated culture (5-week experiment; 66 days)</td>
<td>11.9</td>
</tr>
</tbody>
</table>

DISCUSSION

MTX-induced leukoencephalopathy is an important clinical problem which commonly presents as a change in personality, ataxia, or seizures. It may progress to severe dementia, a comatose state, or death. The pathogenesis of this demyelinating disorder is not known. Clinical reviews indicate that several factors increase the risk of developing leukoencephalopathy (9, 13, 15). Sustained elevations of CSF concentrations of MTX have been associated with the rapid development of leukoencephalopathy. This delayed clearance of MTX from CSF can result from abnormalities in CSF flow. Ventricular outflow obstruction causes increased exposure of periventricular brain to MTX and has been associated with markedly accelerated development of leukoencephalopathic changes in the periventricular white matter (5). This and other CSF flow abnormalities which result in delayed ventricular clearance of MTX are common in patients with carcinomatous meningitis (16). Cranial irradiation administered before or with i.t. and high-dose systemic infusions of MTX has also been associated with an increased incidence of leukoencephalopathy. Patients who receive all three treatment modalities (radiotherapy and i.v. and i.t. MTX) are at the highest risk for developing leukoencephalopathy (9).

Three hypotheses have been proposed to explain the development of leukoencephalopathy. First, vascular injury from radiation and/or chemotherapy has been postulated to result in tissue necrosis and ischemia secondary to occlusion of small and medium size blood vessels (17). A second hypothesis proposes that the primary target of toxicity is the oligodendrocyte, which is responsible for producing and maintaining the myelin sheath (18). The third hypothesis suggests that cellular damage from radiation or chemotherapeutic treatment causes the release of antigens, which lead to the development of an autoimmune response resulting in damage to the myelin sheath and blood vessels (19).

Our study supports a different hypothesis for the mechanism of MTX-induced leukoencephalopathy. The histological and electron microscopic changes reported in this paper using cerebellar explant cultures suggest that the neuron is the primary target of MTX. This neuronal toxicity results in an axonopathy and the delayed demyelination occurs as a secondary event. Our histological and electron microscopic observations are supported by measurements of oligodendroglial cell function by determining CNP activity. The activity of this enzyme has been localized in the cell membrane of the oligodendroglial cell and in the myelin sheath (20). Its activity has been correlated with the ability of the oligodendroglial cell to produce and maintain myelin (21). CNP activity did not significantly change in the cultures treated with MTX, despite the widespread axonal loss which had occurred. In addition, axonal loss was seen in the cultures after 2 weeks of exposure to MTX, whereas the increase in concentrations of MBP in the media was not noted until after the 3rd week of exposure. These findings suggest that the axonopathy led to a secondary loss of myelin, releasing MBP into the media. The toxic changes observed in this culture system occurred in the absence of a vascular system or an intact immune system. These observations, coupled with the apparent sparing of oligodendroglial cell function in the in vitro system, point to different pathogenic mechanisms than have been previously proposed.

Although the cerebellar explants treated with MTX reproduce the histological changes seen with leukoencephalopathy, the development of demyelination in the culture system occurs
more quickly than in patients. There are several possible explanations for the rapid development of this neurotoxicity. First, the cultures were treated with MTX at a relatively high and continuous concentration for the course of the experiment. In patients, MTX administered into cerebrospinal fluid is rapidly cleared by the bulk flow of the cerebrospinal fluid, while in the culture system the only decrease in MTX concentration occurs as a result of cellular metabolism and compound breakdown. Secondly, i.t. administration of MTX in patients results in a variable exposure of brain tissue to MTX, with a gradient of exposure from the ependymal lining into the brain parenchyma (22). In the culture system, there is uniform exposure of the cells to MTX because of the relative thinness of the cultures, which is necessary to maintain cellular viability. Thirdly, there appears to be a significant correlation between the maturation of the central nervous system and sensitivity to MTX. Younger patients are more likely to develop leukoencephalopathy than older patients (23). Although myelination occurs in the culture system, full physiological maturation is not achieved during the 3 weeks of growth prior to the beginning of exposure to MTX. The combination of high and sustained MTX concentrations, diffuse penetration of MTX in the cultures, and the relative immaturity of the cultured neurons probably all contribute to the accelerated development of neurotoxic changes in response to MTX exposure.

The mechanisms involved in the development of neurotoxicity from antineoplastic agents have been difficult to understand because of the complexity of the nervous system and the lack of appropriate laboratory models. Cerebellar explant cultures permit sequential studies of toxin-induced changes with a limited number of experimental variables. Our work using this culture system has led us to believe that MTX primarily causes toxicity to neurons resulting in an axonopathy. The characteristic loss of myelin is a consequence of the neuronal damage. Future experiments will define the effect of radiation treatment combined with MTX and evaluate the mechanisms of toxicity of other antineoplastic agents which are known to be neurotoxic.

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