Survival of Tumor-bearing Mice Exposed to Heavy Water or Heavy Water plus Methotrexate

Jean A. Laiissue, Heinz Bürki, and Willi Berchtold

ABSTRACT

Moderate body deuteration combined with a cytostatic drug [methotrexate (MTX)] significantly increases the survival time of young adult DBA/2 mice bearing transplantable P815, L5178Y, or Li210 tumors. Neoplastic cells were grown in vitro from tumor stock and injected i.p. into mice from two groups, one drinking tap water, the other drinking 30% heavy water in tap water. One-half of the animals in each of these two groups was given a single injection of MTX (4 mg/kg body weight) on 3 consecutive days per week. At death, extension of primary and metastatic tumors was examined and was found to be macro- and microscopically comparable in the corresponding groups. The mean survival time of untreated mice drinking tap water was about 2 weeks following injection of the fast-growing P815, L5178Y, or Li210 tumors and approximately 5 weeks after injection of cells from a slower-growing Li210 subline. Body deuteration alone roughly doubled the survival time solely of mice bearing this Li210 subline. Treatment with MTX approximately doubled the mean survival time of hosts bearing one of the fast-growing tumors. Combined treatment with heavy water and MTX increased the mean survival time of the mice in all groups by 15 to 125% as compared to control values. The reasons for this effect are unknown. However, heavy water has been shown to exert antimitotic activity and to depress the incorporation of radioactive precursors into DNA of proliferating mammalian cells. The depression of antibody formation following antigenic stimulation and the reduction in numbers of nonneoplastic lymphoid cells of mice following moderate body deuteration may have contributed to the enhancement of MTX activity in addition to other effects of deuteration.

INTRODUCTION

In the wake of the discovery of deuterium (3H, D), the natural heavy stable isotope of hydrogen, by Urey et al. (42) in 1932, the biological effects of heavy water (D2O) were examined in a host of experimental situations. While substitution of H2O in the body fluids with D2O in excess of 35% is not compatible with life in mice, rats, and dogs for longer periods of time (8, 21, 26, 40), animals drinking 30% D2O with corresponding deuteration levels of about 24% in their body fluids have a normal life span (22). Similar deuteration of the drinking fluid of tumor hosts results in growth inhibition of transplantable murine neoplasms (Table 1). Deuteration of the animals prior to tumor implantation or in the course of tumor induction by viruses (37) elicits a marked growth inhibition, whereas survival is usually not prolonged. We have found one preliminary report in the literature concerning effects of heavy water combined with cytostatic drugs on transplantable murine neoplasms in vivo (10). Deuteration and drugs lead to a more important reduction of tumor growth than do either of these agents alone. However, death of the hosts often occurs earlier in animals given the combined treatment. In the present study, the effects of body deuteration combined with repeated injections of MTX resulted in prolonged survival of mice bearing various rapidly growing transplantable tumors. A tumor subline with a slower growth rate was sensitive to treatment by body deuteration alone.

MATERIALS AND METHODS

Tumor Cell Lines and Tissue Culture Media. Cells of a mastocytoma, P815, adapted to suspension culture (36) were cultivated in Eagle’s basal medium (Gibco Europe, Glasgow, Scotland), supplemented with NaHCO3 (1 g/liter), folic acid (9 mg/liter), penicillin G (50 mg/liter; Pfizer, Brussels, Belgium), and Mycoplasma-screened horse serum (100 g/liter; Gibco). Lymphoma L5178Y cells were maintained in Dulbecco’s minimum essential medium (Gibco), supplemented with L-glutamine (584 mg/liter; Gibco), NaHCO3 (3.7 g/liter), penicillin G (50 mg/liter; Pfizer), streptomethane (50 mg/liter; Protochemie, Glarus, Switzerland), and 100 ml horse serum (Gibco). Cells of lymphoma Li210 (V) and of a lymphoma Li210 subline that had been maintained in vitro for long periods of time were cultivated in media of the same composition as that used for L5178Y cells. These metastasizing tumors of DBA/2 origin are immunogenic. The mastocytoma and the L5178Y lymphoma had been induced by methylcholanthrene.

Animals. Specific-pathogen-free female DBA/2 mice, 6 to 8 weeks old at the start of the experiment, were obtained from Bomholtgard, Copenhagen, Denmark. They were housed in groups of 5 in cages of the shoebox type with wood bedding (Weichholzgranulat Typ TE 1/2; Gabriel Schill, Muttenz, Switzerland) and were given standard mouse chow (Mäuse- und Rattenfutter Alleinfutter No. 850; NAFAG, Gossau, Switzerland). The cages were kept in a fully air-conditioned room with an artificial light period from 7 a.m. to 7 p.m. The mean room temperature was 21°, and the mean relative humidity was 60%. The mice were weighed every morning.

Drinking Fluid. The tritium contamination of the batch of heavy water with 99.8% deuterium (Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland) was determined in a liquid scintillation counter and was found to be 96 KBq/liter, i.e., at least 10 times less than the maximum permissible concentration in water for occupational exposure in humans (18). Limited amounts of 30% D2O (w/w) were prepared with fresh tap water.

MTX. MTX was chosen for its wide range of applicability in the treatment of animal and human neoplasms. It is thought to exert cytotoxicity by causing depletion of intracellular folates required for the de novo synthesis of thymidylate and purines (13). A single dose of 5
mg/kg body weight affects the proliferative activity of L1210 tumor cells and of normal cell renewal systems of the tumor host. Bone marrow cells recover fully within 6 hr of drug application, and gastrointestinal mucosal cells recover within 24 hr, as measured by incorporation of radioactive DNA precursors. Conversely, the proliferative activity of the L1210 cells still remains reduced to a few % of the initial value 24 hr after MTX (6). The choice of the MTX dose for the present study was based on these observations. Prolonged survival of L1210-bearing mice has been obtained by injections of MTX every fourth day with a dose equivalent to a daily dose of 3.6 mg/kg body weight, although continuation of treatment for long periods of time resulted in chronic toxicity and death (12). The sensitivity to MTX of various neoplastic cell lines and sublines, however, varies greatly and appears to be related to the uptake of the drug by neoplastic cells (24).

The dry substance, provided in ampuls containing 50 mg (Lederc Methotrexate), was dissolved with sterile aqueous NaCl solution (0.72 g/liter) to a final concentration of 500 mg/liter. Single injections of 4 mg/kg body weight, corresponding to 0.2 ml for a 25-g mouse, were given i.p. on 3 consecutive days per week between 5 p.m. and 6 p.m., beginning 1 day after tumor inoculation.

Methods

Tumor Transplantation Procedures. Aliquots of the stock tumor cell suspension, kept frozen at -80°C, were thawed 2 weeks prior to injection and maintained in suspension cultures. Since the survival time of tumor hosts is also related to the number of injected cells, inoculum sizes of 100,000 cells were chosen for the rapidly growing tumors and of 400,000 cells for the slower-growing L1210 subline. On Day 0, the cell numbers were brought to the selected concentration. Cell counts were obtained at the beginning and end of the injection period, and the data in Table 2 indicate the mean of these values. The cultures were kept in spinner vials, and 0.2 ml of the cell suspension was injected into the peritoneal cavity of the recipient mice, using the same insertion point of the needle in the lower one-third of the abdomen in all animals (29). The viability of the cells in the suspension was monitored by the point of the needle in the lower one-third of the abdomen in all animals.

Experimental Subgroups. Mice to be inoculated with a given cell line (Table 2) were divided into the following subgroups: Subgroup 1, mice drinking tap water (H2O); Subgroup 2, mice drinking 30% heavy water in tap water (30% D2O); Subgroup 3, mice drinking tap water and treated with MTX; Subgroup 4, mice drinking 30% D2O and treated with MTX. In the second experiment with L1210 cells, MTX was not used; the subgroups comprised mice drinking tap water or 7.5%, 15%, or 30% D2O, respectively (Table 2).

Morphological Examination. All mice were dissected with as little delay as possible after death. The extension of the tumor was recorded macroscopically. Samples of major tumor sites and of the sternum, spine, or femurs were fixed in at least 10 volumes of neutral buffered formalin and processed for histology.
The growth rate, however, is considerably slower than that of the tumors described above. Untreated mice died within 10 weeks after L1210 tumor cell inoculation versus 2 to 3 weeks following inoculation of the faster-growing P815, L5178Y, or L1210 (V) cells. Addition of 30% D₂O to the drinking fluid of L1210 lymphoma-bearing mice yielded a significant prolongation of survival time \( (P < 0.0005; \text{Chart 3, top}) \). In contrast to the observations made in the previous experiments, prolongation of survival time brought about by 30% D₂O was greater than that resulting from treatment with MTX (Table 3). However, the difference in survival time between animals treated with heavy water alone and those treated with heavy water and MTX was significant \( (P < 0.0005) \).

Concentrations of D₂O in the drinking fluid below 30% also resulted in a marked prolongation of survival of mice bearing L1210 lymphoma (Chart 3, bottom; Table 3). The difference in survival time between mice drinking 7.5 or 30% heavy water was not significant \( (P = 0.3) \).

**Morphological Examination.** All animals in every experimental group displayed a widespread tumor dissemination. There was an extensive involvement of peritoneal sites by the primary ascites tumor. In addition, lymph nodes, pancreas, intestines, liver, ovaries, kidneys, adrenals, spleen, lungs, thymus, and bone marrow were often infiltrated and/or partially destroyed by tumor tissue. Conventional histological examination failed to reveal obvious differences in extent of tumor involvement in primary and metastatic sites and in extent of tumor necrosis between control mice and those treated with 30% D₂O and/or MTX. Within a single tumor, the number of mitotic figures markedly differed in various areas, e.g., in central versus peripheral zones, or in primary versus metastatic sites. Moreover, it was impossible to observe the same time interval between death of the mouse and fixation of organ samples. Therefore, mitotic counts were not done.

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**Table 3**

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Survival time (days)</th>
<th>Subgroups compared</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H₂O (Subgroup 1)</td>
<td>30% D₂O (Subgroup 2)</td>
<td>H₂O/MTX (Subgroup 3)</td>
</tr>
<tr>
<td>Mastocytoma P815</td>
<td>13.2 ± 1.0</td>
<td>13.0 ± 1.1</td>
<td>29.6 ± 4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30.4 ± 3.8</td>
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<td></td>
<td></td>
<td></td>
<td>17.3 ± 1.5</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>39.3 ± 9.0</td>
</tr>
<tr>
<td>Lymphoma L5178Y</td>
<td>16.6 ± 1.6</td>
<td>19.2 ± 1.9</td>
<td>30.4 ± 3.8</td>
</tr>
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<td></td>
<td></td>
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<td>17.3 ± 1.5</td>
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<td>39.3 ± 9.0</td>
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<td></td>
<td></td>
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<td>30% D₂O/MTX</td>
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<tr>
<td>Lymphoma L1210 (V)</td>
<td>15.0 ± 1.0</td>
<td>17.5 ± 1.8</td>
<td>29.6 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>15.0 ± 1.8</td>
<td>17.5 ± 1.8</td>
<td>30.4 ± 3.8</td>
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<tr>
<td></td>
<td>30% D₂O/MTX</td>
<td>30% D₂O/MTX</td>
<td>17.3 ± 1.5</td>
</tr>
</tbody>
</table>

\( ^a \) Mean ± S.D.

\( ^b \) Subgroups with one long-term survivor.

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**Chart 1.** Relative number (%) of surviving mice following i.p. injection of P815 mastocytoma cells on Day 0. C, untreated control mice; A, mice drinking 30% D₂O in H₂O, but otherwise untreated; O, mice drinking tap water and given i.p. injections of MTX (4 mg/kg body weight) on 3 consecutive days per week. A, mice drinking 30% D₂O and given i.p. injections of MTX (4 mg/kg body weight).

**Chart 2.** Top, relative number of surviving mice following i.p. injection of L5178Y lymphoma cells on Day 0. Symbols as in Chart 1. Bottom, surviving mice versus time following i.p. injection of L1210 (V) lymphoma cells on Day 0. Symbols as in Chart 1.
indices of renewing nonneoplastic mammalian cells, e.g., of small intestinal crypt epithelia of mice (26). In contrast, these epithelia display normal mitotic indices in mice drinking 30% D₂O. ¹ The few available preliminary data fail to indicate a significant effect of moderate deuteration on mitotic activity of neoplastic mammalian cells in vivo (9).

Highly deuterated sea water depresses the uptake of radioactive thymidine by the cell and its incorporation into DNA of fertilized sea urchin eggs (14). The very few experimental studies indicate a similar effect in mammals (e.g., Refs. 7, 25, and 35). There are no available data on the effects of heavy water on the incorporation of radioactive DNA precursors by neoplastic mammalian cells. The effect of deuterium on tumor cells in vivo may also be due to cell destruction. Increased numbers of nonviable tumor cells have been observed in the ascites of tumor-bearing mice treated with heavy water (9). In the present study, however, tumor necrosis was seen in all experimental groups.

The effects of moderate body deuteration on the survival of tumor hosts may have been mediated through isotope effects. Substitution of ¹H for ²H with a change in mass ratio by factor of 2, the largest for any pair of stable isotopes of the same element, induces complex biological changes. They arise through replacement of ¹H by deuterium in solvents and in organic molecules, particularly in biopolymers such as proteins and DNA. The higher melting and boiling points, the higher density and viscosity, and the poorer solvent properties for salts and gases of heavy water as compared to H₂O may affect a host of biochemical reactions (21), with complex repercussions on intra- and intercellular interactions. The smaller self-ionization of heavy water versus H₂O makes pD different from pH (21). Thereby, pharmacokinetic parameters of MTX might in turn be affected, such as pH-dependent ionization, solubility, and binding to dihydrofolate reductase (4); binding of MTX to plasma proteins (6, 34); uptake of the drug by neoplastic cells (24); clearance of MTX from blood plasma (38) or from ascites induced by i.p. tumor growth; and renal filtration and secretion of MTX (41).

The faster a cell population is proliferating, the more susceptible it is to the lethal effects of a variety of antimetabolites (16). Paradoxically, the slower-growing L1210 tumor was very sensitive to treatment with heavy water alone, whereas the faster-growing tumors were not. This indicates the involvement of other than antimetabolic interferences mediated by deuterium which may require a prolonged exposure to heavy water. The course taken by a chemically induced, weakly immunogenic, malignant neoplasm in vivo may be influenced by antineoplastic agents and by the immunological response of the host (11, 28, 32, 33). Heavy water exerts immunosuppressive effects. Moderate body deuteration of young adult mice results in dose-dependent depression of specific antibody production following stimulation with tetanus toxoid (26). MTX also depresses humoral antibody formation and blocks manifestations of delayed hypersensitivity (39). Although immune suppression often results in enhanced tumor growth and earlier death of tumor hosts, some transplantable tumors may grow more slowly in hosts with deficient suppressor cell activity or deficient production of antibodies to tumor cell antigens (31). To date, there are no available data on the effects of heavy water on

¹ A. Hodel and J. A. Lais, unpublished observations.
lymphoid cell subpopulations to support the existence of such a hypothetical mechanism. However, the antineoplastic effect of heavy water alone or combined with cytostatic drugs and its minor toxicity at effective antineoplastic concentrations appear remarkable enough to call for further investigations.

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