Effects of Early Chemotherapeutic Treatment on Learning in Adolescent Mice: Implications for Cognitive Impairment and Remediation in Childhood Cancer Survivors

Emily B. Bisen-Hersh, Philip N. Hineline, and Ellen A. Walker

Abstract

**Purpose:** Among children diagnosed with acute lymphoblastic leukemia (ALL) and given chemotherapy-only treatment, 40% to 70% of survivors experience neurocognitive impairment. The present study used a preclinical mouse model to investigate the effects of early exposure to common ALL chemotherapeutics methotrexate (MTX) and cytarabine (Ara-C) on learning and memory.

**Experimental Design:** Preweanling mouse pups were treated on postnatal day (PND) 14, 15, and 16 with saline, MTX, Ara-C, or a combination of MTX and Ara-C. Nineteen days after treatment (PND 35), behavioral tasks measuring different aspects of learning and memory were administered.

**Results:** Significant impairment in acquisition and retention over both short (1 hour) and long (24 hours) intervals, as measured by autoshaping and novel object recognition tasks, was found following treatment with MTX and Ara-C. Similarly, a novel conditional discrimination task revealed impairment in acquisition for chemotherapy-treated mice. No significant group differences were found following the extensive training component of this task, with impairment following the rapid training component occurring only for the highest MTX and Ara-C combination group.

**Conclusions:** Findings are consistent with those from clinical studies suggesting that childhood cancer survivors are slower at learning new information and primarily exhibit deficits in memory years after successful completion of chemotherapy. The occurrence of mild deficits on a novel conditional discrimination task suggests that chemotherapy-induced cognitive impairment may be ameliorated through extensive training or practice.

Introduction

Advancements in treatment of acute lymphoblastic leukemia (ALL), the most common form of cancer in children and adolescents, have led to a remission rate of more than 90% among patients (1). Furthermore, the substitution of intrathecal chemotherapy for cranial irradiation has reduced adverse late effects resulting from treatment (2, 3). However, 40% to 70% of childhood cancer survivors given chemotherapy-only treatment will experience neurocognitive deficits years later, including impairments in attention, working memory, and processing speed (4–6).

Preclinical models have been used to directly test the effects of chemotherapeutic agents on learning and memory (see ref. 7 for review). The vast majority of these studies have focused on treatment of adolescent or adult rodents, whereas a few have assessed the long-term impact of preweanling treatment at postnatal day (PND) 15 (8) or PND 17 (9–12). As models using adult rodents are not adequate for studying the emergent effects of childhood chemotherapy treatment, additional examination of long-term impairment following early exposure to chemotherapeutics is warranted. Of the chemotherapeutic agents used in ALL treatment, methotrexate (MTX) remains the most studied because of its known neurotoxic effects, which include antifolate disruption of DNA synthesis, reduction of hippocampal cell proliferation and blood vessel density, as well as degeneration of white matter and white matter necrosis (13–16). Following treatment with MTX alone, behavioral disruptions have been noted on tasks of learning and memory (8, 10, 15, 17 but see ref. 18).

MTX is commonly administered with cytarabine (Ara-C) during intrathecal chemotherapy for ALL. In cell culture, Ara-C alone produces death of oligodendrocytes and gliarestricted precursor cells (19). In vivo, Ara-C alone disrupts cell division in mice (19) and induces retraction of the apical dendrites of the anterior cingulate cortex in rats (20). Memory loss and white matter changes have also been reported in patients following treatment with Ara-C (21).
The current study was to characterize emergent learning and evidence in rodents for long-term spatial memory impairment has been found (20). Although MTX and Ara-C are commonly administered simultaneously or sequentially owing to their synergistic effects on cancer cells (22), the impact of this combination treatment on cognitive impairment has not been examined.

The present study tested male and female mice using 3 assays of learning and memory: an autoshaping procedure, a novel object recognition task, and a novel conditional discrimination task. The autoshaping procedure provides a rapid and objective measure of whether a drug affects acquisition or retention of a learned response (23, 24). The novel object recognition task involves the spontaneous exploration of objects and is based on the well-documented fact that rodents tend to explore novel stimuli more than familiar ones. This one-trial learning paradigm uses delay-dependent behavior to model memory in a rodent (25). Conditional discrimination involves the use of conditional information to inform goal-directed performance (26). Choice of one of the simultaneously present stimuli is reinforced only when one of the additional conditional stimuli is present, whereas choice of the other simultaneous stimulus is reinforced only when the second conditional stimulus is present (27). The current procedure used a conditional temporal discrimination, in which response accuracy was contingent on either a short or long stimulus duration. The novel procedure was validated using a well-known amnesic agent, scopolamine (28), before conducting this study (Supplementary Fig. S1). The aim of the current study was to characterize emergent learning and memory deficits during adolescence induced by preweaning treatment with either MTX or Ara-C, administered alone or in combination. Because the autoshaping procedure and novel object recognition task both involve one-trial rapid learning paradigms, the conditional discrimination procedure was developed to include an extensive training component presented before a rapid training component. A second aim of this study was to compare the effect of extensive versus rapid training on response accuracy, thus to examine whether practice would reduce the extent of learning impairments observed following early exposure to chemotherapeutics.
Drugs
MTX (Hospira, Inc.) and Ara-C (Sigma-Aldrich) were reconstituted with saline. Injections were administered to preweanling mouse pups in a volume of 0.1 mL/10 g intraperitoneally (i.p.) on PND 14, 15, and 16. Pups were individually weighed before the injection. Immediately following the injection, pups were returned to the dam and litter. In this manner, the pups were only removed from the dam for approximately 2 minutes to minimize stress. Each dose of MTX (1 or 2 mg/kg), Ara-C (10 or 20 mg/kg), or the MTX and Ara-C combination (1 mg/kg MTX and 10 mg/kg Ara-C or 2 mg/kg MTX and 20 mg/kg Ara-C) was administered in a separate group of mice (between-groups design). MTX and Ara-C were stored and handled in accordance with guidelines set forth by the Temple University Department of Environmental Health and Radiation Safety.

To estimate the appropriate treatment doses and regimens for MTX and Ara-C, allometric techniques were used to convert human dosing schedules to comparable adult mouse schedules and temporal comparisons of chronic dosing were made with the pharmacokinetic equation for maximum lifespan potential (30, 31). Because pharmacokinetic data are not available for preweanling mice and adult doses of MTX and Ara-C are approximately 10-fold higher than childhood doses, the preweanling pup dose was reduced by 10-fold.

Experiment 1: autoshaping task
Apparatus. Twelve experimental chambers, as previously described (21.6 cm × 17.8 cm × 12.7 cm, Model ENV-307W, MED Associates), were used for the autoshaping procedure (18).
Procedure. The autoshaping procedure was modified from the method previously described for mice by Vanover and Barrett (23) by omitting the noncontingent dipper presentation (32). At PND 34, mice (n = 82) were separated into individual cages, weighed, and food-restricted for 24 hours before day 1 acquisition (PND 35). Water remained available ad libitum. On day 1 of the autoshaping procedure, each mouse was weighed and placed inside the experimental chambers for 15 minutes before its session started. During each session, the house light illuminated the chamber and a tone occurred on a variable-time schedule (mean of 45 seconds; range, 4–132 seconds). The tone remained on for 6 seconds or until a nose-poke response occurred. If the mouse made a center-hole nose-poke during the tone, a 0.01- cm³ dipper filled with a vanilla-flavored liquid nutritional drink Ensure Plus/water (50:50) solution was presented for 3 seconds in the center-hole nose-poke, and the tone was turned off. Nose-poke responses in the absence of the tone were counted but had no programmed consequences. Each session lasted for 2 hours or until 20 reinforced nose-pokes were recorded. Mice were fed 3 g of food and returned to their cages. On day 2, the procedure was repeated.
Data and statistical analysis. Each nose-poke into the center nose-poke hole in the presence of the tone resulted in presentation of the dipper of Ensure solution and was recorded as a reinforced response (up to a maximum of 20 in each 2-hour session). The main measure of acquisition and retention was the mean-adjusted latency. Mean-adjusted latency was the elapsed time, in seconds, to the 10th reinforced response minus the latency to the first reinforcer (L10 - L1). To calculate the rate of dipper nose-pokes responding, the total number of dipper nose-pokes made during the session—regardless of the presence or absence of the tone—was recorded and divided by the total session time in seconds for each mouse. This dipper rate measure served as a guide to whether differences in adjusted latency were dependent on overall rate of responding. Nose-pokes in the left and right holes were not reinforced but were counted and divided by total session time as a measure of response discrimination. Data from all mice were included in acquisition measures from the day 1 session. However, mice that failed to achieve at least 10 reinforcers on day 1 of testing were excluded from the mean latency measures on day 2. The rationale for this exclusion was that it was not appropriate to evaluate day 2 retention of a response that had been insufficiently reinforced, or not reinforced at all, on day 1 (23, 24).

For the autoshaping procedure, the effect of treatment on day 1 mean-adjusted latency was compared using a one-way ANOVA followed by Dunnett multiple comparison post hoc tests. To analyze within-group differences between day 1 and 2 on mean-adjusted latency, dipper response rates, and nonreinforced nose-poke response rates repeated measures ANOVAs were used. To analyze sex differences within each treatment group on all measures, independent t tests were used. Significance was set at P < 0.05 for all statistical tests.

Experiment 2: novel object recognition
Apparatus. For assessments of novel object recognition, the testing cages were identical to those in which mice were housed in the animal facility. The objects used during testing were Lego figures of equal size and material that differed in design. These were weighted to minimize the movement of objects by mice during trials. Trials were video recorded and later coded by an experimenter who was blind to treatment groups.
Procedure. The objects to be used were first assessed to ensure that there were neither intrinsic preferences nor aversions and that each object would be explored for similar durations upon initial exposures. Exploration was defined as directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose or mouth. Rearing up on the object was counted only if facing toward, but not away from, the object (33, 34).

An individual mouse injected on PND 14, 15, and 16 but naïve to behavioral testing (n = 84) was placed in a testing cage on PND 35 for a 20-minute acclimation period and then returned to its home cage for 30 minutes. Each mouse was then placed in the same testing cage for trial 1. The testing cage contained 2 identical objects placed in the left and right corners at the opposite end of the cage from where the mouse entered. A mouse was always placed in the testing
cage facing away from the objects. The amount of time spent exploring each object (defined earlier) was recorded over a 5-minute period. At the end of trial 1, mice were placed back into the home cage for the duration of the 1-hour delay period. Following the 1-hour delay, each mouse was returned to the same testing cage for trial 2, which contained one of the previous objects (a familiar object) and a novel object, placed in the same locations. The amount of time spent exploring each object was recorded over a 5-minute period. Familiar and novel objects, as well as locations of objects, were counterbalanced between subjects.

Data and statistical analysis. To assess variability in exploration, total exploration time for both familiar and novel objects was compared between groups. To measure differences in exploration of a familiar versus a novel object after a 1-hour delay period, exploration time of the novel object was expressed as a percentage of the exploration time of a familiar object as outlined below:

Discrimination index (\%) = \frac{[\text{novel} (s)/\text{familiar} (s)]}{100}

In this measure, 100% indicates an equal amount of time spent exploring the familiar and novel objects, less than 100% indicates more time spent exploring the familiar object, and more than 100% indicates more time spent exploring the novel object (as described in ref. 15).

For the novel object recognition procedure, the effect of treatment on novel object discrimination and total exploration was compared using one-way ANOVAs followed by Dunnett multiple comparison post hoc tests. To analyze sex differences in novel object discrimination within each treatment group, independent t tests were used. Significance was set at \( P < 0.05 \) for all statistical tests.

Experiment 3: conditional discrimination

Apparatus. The same 12 experimental chambers described for the autoshaping procedure were used for the conditional discrimination procedure, with the addition of stimulus lights above each poke hole (ENV-321M).

Procedure. The conditional discrimination procedure used a tone/light compound stimulus and temporal duration and was modified from other conditional discrimination tests used in rodents (26, 35). At PND 34, the mice injected on PND 14, 15, and 16 were separated into individual cages, weighed, and food-restricted for 24 hours before the start of experimental testing. Water remained available ad libitum. On day 1 of training, each mouse was weighed and placed inside an experimental chamber for 15 minutes before the session started. During each session, the house light illuminated to indicate the availability of a vanilla-flavored liquid nutritional drink, Ensure Plus/water (50:50) solution, which would be presented in a 0.01-cm³ dipper. These food deliveries were contingent upon correct responding, as defined separately for each phase described below. Each session lasted for 1 hour, following which mice were fed 3 g of food and returned to their cages.

The conditional discrimination procedure involved a series of 8 phases, and specific criteria were completed for each before progressing to the next phase. “Extensive training” was defined as completion of phases I–V, followed by a test in which a discrimination ratio was measured (phase VI). "Rapid training" was defined as phase VII, followed by a test in which a discrimination ratio was measured (phase VIII). Details of each phase are outlined in the Supplementary Materials. Briefly, during phases I and II a nose-poke response into the left or right hole, respectively, was reinforced on a fixed-ratio (FR-1) schedule of reinforcement. During phases III and IV, an audible tone was presented along with an illuminated stimulus light above the left or right poke hole for 2 or 8 seconds, respectively, and a nose-poke response into the correct hole was reinforced. During phase V, the duration of the tone/light cue was randomly alternated between short (2 seconds) and long (8 seconds), and a nose-poke response into the correct hole was reinforced. Mice were trained on this set of contingencies to 70% correct responding over 2 consecutive sessions before the discrimination ratio was assessed during the phase VI test session. During phase VII, the contingencies previously described were reversed, and this new set of contingencies was trained for 1 session before the discrimination ratio was assessed during the phase VIII test session.

Data and statistical analysis. Acquisition was measured by the number of sessions required to reach phase I criteria, defined as 20 reinforced responses in the active nose-poke hole, with the total responses including at least 75% active nose-poke hole responding. To examine the effect of treatment on acquisition, a one-way ANOVA was used, followed by Dunnett multiple comparison post hoc tests. The main measure of performance on the conditional discrimination task was the discrimination ratio (26), defined as

\[
\text{Discrimination ratio} = \frac{\text{correct responding}}{\text{correct responding} + \text{incorrect responding}} \times 100
\]

The discrimination ratio was calculated for each mouse at phase VI and VIII to measure response accuracy following extensive and rapid training, respectively. Only mice that reached the 70% criteria at phase V were included in the following analyses. A repeated measures ANOVA was used to assess the effect of treatment and training (extensive or rapid) on correct discrimination. Separate one-way ANOVAs with Dunnett multiple comparison post hoc tests were used to examine treatment effects within each training type (extensive or rapid). Significance for all statistical tests was set at \( P < 0.05 \).

Results

Experiment 1: autoshaping task

Group composition for the autoshaping task is shown in Supplementary Table S1 \((n = 82)\). Sex differences within each treatment group revealed a significantly higher dipper response rate \([f(10) = 2.76; P < 0.05]\) on day 2 for male mice treated with saline compared with female mice treated with saline, although there was no effect on mean-adjusted latency \((P > 0.05)\). Among mice treated with 2 mg/kg MTX, there was a significantly shorter mean-adjusted latency \([f(10) = 2.76; P < 0.05]\) and higher dipper response rate.
Early Chemotherapy Impairs Learning in Adolescent Mice

On day 2, the mice were placed back into the experimental chambers, and the experimental conditions were identical to day 1. As shown in Fig. 2A, mice treated with saline consistently responded in the dipper in the presence of the tone, with a mean-adjusted latency that was significantly shorter than on day 1 \( F(1,10) = 6.71; P < 0.05 \), as revealed by a repeated measures ANOVA. As shown in Fig. 2B and C, respectively, the dipper response rate was significantly increased on day 2 compared with day 1 \( F(1,11) = 25.82; P < 0.001 \) and the nonreinforced response rate was significantly decreased on day 2 compared with day 1 \( F(1,11) = 10.68; P < 0.01 \). In summary, mice treated with saline responded with a significantly faster latency and dipper response rate and directed fewer of their responses to the nonreinforced nose-poke holes on day 2, showing that the dipper nose-poke response had been effectively learned on day 1 and retained on day 2.

**Day 1 acquisition.** Compared with saline controls (1,179 ± 176 seconds), mice treated with 2 mg/kg MTX (3,226 ± 757 seconds), 1 mg/kg MTX and 10 mg/kg Ara-C (3,099 ± 639 seconds), or 2 mg/kg MTX and 20 mg/kg Ara-
C (2,957 ± 603 seconds) took significantly longer to obtain the liquid reinforcers on day 1 (Fig. 2A), showing an impairment in acquisition of a learned nose-poke response ($P < 0.01$).

**Day 2 retention.** Mice treated with 10 mg/kg Ara-C [$F(1,11) = 7.79; P < 0.05$] with 20 mg/kg Ara-C [$F(1,10) = 7.80; P < 0.05$] or with 1 mg/kg MTX and 10 mg/kg Ara-C [$F(1,11) = 6.76; P < 0.05$] responded with significantly faster mean-adjusted latencies on day 2 compared with day 1 (Fig. 2A). This shows that the dipper nose-poke response had been effectively learned on day 1 and retained on day 2. However, mice treated with 1 mg/kg MTX, 2 mg/kg MTX, or 2 mg/kg MTX and 20 mg/kg Ara-C displayed equal latencies on both days, showing impairment in retention of a learned nose-poke response, specifically that of responding in the dipper well in the presence of the tone ($P > 0.05$).

Only mice treated with the 1 mg/kg MTX and 10 mg/kg Ara-C combination displayed equal dipper response rates on both days ($P > 0.05$). Mice treated with 1 mg/kg MTX [$F(1,10) = 8.13; P < 0.05$] with 2 mg/kg MTX [$F(1,11) = 16.46; P < 0.01$], 10 mg/kg Ara-C [$F(1,11) = 26.15; P < 0.001$], 20 mg/kg Ara-C [$F(1,10) = 13.40; P < 0.01$], or 2 mg/kg MTX and 20 mg/kg Ara-C [$F(1,11) = 5.94; P < 0.05$] displayed a significantly increased dipper response rate on day 2 compared with day 1 (Fig. 2B). That is, for these mice a greater amount of time was spent poking in the center hole, a response that had been reinforced during the previous session. Therefore, in these groups, it is unlikely that differences in adjusted latency were due to impairments in overall rate of responding.

Mice treated with 1 mg/kg MTX [$F(1,10) = 14.54; P < 0.01$], 2 mg/kg MTX [$F(1,11) = 6.64; P < 0.05$], 10 mg/kg Ara-C [$F(1,11) = 14.49; P < 0.01$], 20 mg/kg Ara-C [$F(1,10) = 50.55; P < 0.001$], 1 mg/kg MTX and 10 mg/kg Ara-C [$F(1,11) = 5.95; P < 0.05$], or 2 mg/kg MTX and 20 mg/kg Ara-C [$F(1,11) = 32.49; P < 0.001$] displayed significantly decreased nonreinforced response rates on day 2 compared with day 1 (Fig. 2C). These results show that all groups of mice directed fewer of their responses to the nonreinforced nose-poke holes on day 2. Therefore, it is unlikely that differences in adjusted latency were due to impairment in response discrimination.

**Experiment 2: novel object recognition**

Of the mice that completed a novel object recognition task, only those that explored each object for more than 10 seconds during trial 1 were included in discrimination index and total exploration time analyses ($n = 81$). As a result, 3 mice from the 20 mg/kg Ara-C group were excluded from these analyses. Group composition for the novel object recognition task is shown in Supplementary Table S1. Given the fact that no significant differences were found between male and female mice within any group, data were collapsed for mice that were given the same saline or chemotherapy treatment.

The discrimination index is displayed in Fig. 3A. A discrimination index of more than 100% indicates that the mouse spent more time exploring the novel object than the familiar one. Thus, with a discrimination index of 183.34 ± 40.88, saline controls spent significantly more time exploring the novel object compared with the familiar one ($P < 0.05$).

A significant effect of preweaning treatment on discrimination index [$F(6,69) = 2.82; P < 0.05$] was observed, with mice receiving the higher dose of MTX (2 mg/kg), Ara-C (20 mg/kg), or a combination (2 mg/kg MTX and 20 mg/kg Ara-C) spending an equal amount of time exploring both objects and showing a significantly lower discrimination index compared with saline controls ($P < 0.05$). No significant differences were found between the 2 mg/kg MTX, the

![Figure 3](clincancerres.aacrjournals.org) Effects of early treatment of MTX and Ara-C on novel object recognition at PND 35: (A) discrimination index and (B) total exploration time. A discrimination of the novel object compared with the familiar object following a 1-hour delay was compared between mice treated with saline, MTX, Ara-C, or an MTX and Ara-C combination. Mice treated with the higher dose of MTX (2 mg/kg), Ara-C (20 mg/kg), or MTX and Ara-C combination (2 mg/kg MTX and 20 mg/kg Ara-C) spent significantly less time exploring the novel object during the recognition trial, compared with saline controls. *, $P < 0.05$. Ordinate, (time spent exploring the novel object/time spent exploring the familiar object) × 100. B, total exploration time of both objects during the recognition trial was compared between mice treated with saline, MTX, Ara-C, or an MTX and Ara-C combination. No significant differences were observed between treatment groups. Ordinate, time spent exploring the novel object/familiar object, in seconds. All abscissa, saline or chemotherapy treatment group (dose in mg/kg, administered i.p.). Values are mean ± SEM.
20 mg/kg Ara-C, and the 2 mg/kg MTX and 20 mg/kg Ara-C groups ($P > 0.05$). Mice receiving the lower dose of MTX (1 mg/kg), Ara-C (10 mg/kg), or a combination (1 mg/kg MTX and 10 mg/kg Ara-C) showed a lower discrimination index compared with saline as well, although these effects did not reach statistical significance (Fig. 3A). No significant differences in average total exploration time were observed (Fig. 3B) between any of the groups ($P > 0.05$). Therefore, it is unlikely that impairment on the discrimination index is due to an unequal amount of time spent exploring the objects during the test trial.

**Experiment 3: conditional discrimination**

Group composition for the conditional discrimination task is shown in Supplementary Table S1. Of the mice that completed the conditional discrimination task, only mice that reached the 70% criteria at phase V were included in statistical analyses ($n = 65$). No significant differences were found between male and female mice within any group; therefore, the data were collapsed for male and female mice that were given the same treatment.

Mice treated with saline or chemotherapeutics were tested at PND 35, 19 days after treatment. As shown in Fig. 4A, there was a significant effect of treatment on acquisition [$F(6,60) = 2.98; P < 0.05$]. In general, chemotherapy-treated mice required a higher number of sessions to reach phase I criteria compared with saline controls, although this effect only reached significance for mice treated with 2 mg/kg MTX or 10 mg/kg Ara-C ($P < 0.05$).

As revealed by a repeated measures ANOVA and displayed in Fig. 4B, the discrimination ratio was lower following rapid training relative to extensive training for all groups [$F(1,58) = 38.13; P < 0.001$]. There was a significant interaction between treatment and training [$F(6,58) = 3.82; P < 0.01$]. Separate one-way ANOVAs for each training type revealed no significant difference in discrimination ratio between groups following extensive training ($P > 0.05$) but a significant difference in discrimination ratio between groups following rapid training [$F(6,58) = 4.78; P < 0.001$]. Dunnett multiple comparison post hoc tests revealed a significantly lower discrimination ratio only in mice treated with the higher combination treatment (2 mg/kg MTX and 20 mg/kg Ara-C) relative to saline controls ($P < 0.05$). In addition, several chemotherapy-treated groups earned fewer reinforcer deliveries following rapid training compared with extensive training, such as mice treated with 2 mg/kg MTX ($P < 0.05$), 10 mg/kg Ara-C ($P = 0.08$), and 2 mg/kg MTX and 20 mg/kg Ara-C ($P < 0.05$). Saline-treated mice earned a comparable number of reinforcers following both types of training ($P > 0.05$).

**Discussion**

In the current study, preweanling mice were treated on PND 14, 15, and 16 with saline, MTX, or Ara-C, injected alone or in combination. Behavioral tests conducted 19 days after treatment revealed impairment on simple, one-trial learning tasks tested once during the adolescence phase of development. More specifically, MTX alone and in combination with Ara-C seemed to be the most deleterious for producing disruptions of acquisition and retention without altering response rates or response discrimination in the autoshaping procedure. In the novel object recognition task, higher doses of MTX and Ara-C, as well as their combination, disrupted short-term recognition memory without altering total exploration time. Findings from the conditional discrimination procedure revealed impairment

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**Figure 4.** Effects of early treatment of MTX and Ara-C on conditional discrimination at PND 35: (A) acquisition and (B) discrimination ratio following extensive and rapid training. A, number of sessions to reach criteria for phase I of the conditional discrimination procedure was compared between mice treated with saline, MTX, Ara-C, or an MTX and Ara-C combination. There was a significant effect of preweanling treatment on acquisition, although only mice treated with 2 mg/kg MTX or 10 mg/kg Ara-C required a significantly higher number of sessions to reach criteria compared with saline controls. *, $P < 0.05$. Ordinate, number of sessions required to reach phase I criteria. Abscissa, saline or chemotherapy treatment group (dose in mg/kg, administered intraperitoneally). B, accuracy in responding was compared between mice treated with saline, MTX, Ara-C, or an MTX and Ara-C combination following extensive and rapid training. There was no significant difference in discrimination ratio between groups following extensive training. There was a significantly lower discrimination ratio in mice treated with the higher combination (2 mg/kg MTX and 20 mg/kg Ara-C), relative to saline controls, following rapid training. *, $P < 0.05$. Ordinate: [correct responding/(correct + incorrect responding)] ×100. Abscissa, training type. Values are mean ± SEM.
in acquisition for chemotherapy-treated mice relative to saline controls, with these groups requiring a higher number of sessions to reach the first set of criteria and move forward with training. Impairment in acquisition and short-term retention are consistent with clinical studies suggesting that childhood cancer survivors are slower at learning new information (36) and primarily show deficits in memory (6, 37). Cognitive late effects are emergent in that they usually present 3 or more years after diagnosis (38).

The main measure of performance on the conditional discrimination task was the discrimination ratio, which was calculated following both extensive and rapid training. There was no difference found between groups following extensive training. This result is not surprising given that mice were trained to 70% correct discrimination before testing, showing that comparable performance can be obtained between saline- and chemotherapy-treated mice even though, initially, it may take longer for the chemotherapy-treated mice to reach this level. Impairment in acquisition was generally found for the chemotherapy-treated mice relative to saline controls, with these groups requiring a higher number of sessions to reach the first set of criteria and move forward with training. Once criteria on phase I were reached, it was unusual for mice to display any further delay in progress through the remainder of the phases (data not shown). The original hypothesis that mice treated with MTX and Ara-C would display impairment following rapid training was not fully supported, however. Overall, all groups had a lower discrimination ratio following rapid training compared with their discrimination ratio following extensive training, but only the 2 mg/kg MTX and 20 mg/kg Ara-C combination group showed a difference in response accuracy compared with saline. This combination group did not display an initial delayed acquisition but later revealed impairment when the original contingencies were reversed. In addition, although only one group displayed significantly lower response accuracy compared with saline controls following rapid training, several chemotherapy-treated groups earned fewer reinforcer deliveries compared with the number earned following extensive training. This finding suggests that chemotherapy-treated mice had a higher number of omitted trials during the rapid training test session compared with the saline group, which earned a comparable number of reinforcer deliveries following both types of training.

To date, there has been a lack of preclinical models to study the development of cognitive late effects after chemotherapy treatment in childhood ALL survivors (39). Thus, a notable aspect of the current study is the use of a developmental framework for assessing the emergent effects of early chemotherapy treatment. This stands in contrast to the growing body of literature focused on models in adult rodents (see refs. 7, 40 for review). For example, the auto-shaping procedure has been used to study cognitive deficits in adult mice produced by single and repeated administration of several chemotherapeutics (18, 41). The fact that MTX alone fails to alter acquisition or retention in adult mice but does have an impact on mice treated at a younger age using an identical procedure emphasizes the importance of a developmental framework when modeling the late effects of childhood ALL treatment. A single administration of MTX in adult rats produced disruption on a novel object recognition task following a 1-hour delay (15). However, results have been inconsistent in the area of discrimination learning, including both Pavlovian and instrumental procedures. In the small number of studies examining the effects of early MTX treatment in rat pups, deficits on conditioned emotional response and conditioned taste aversion tasks have been noted in some (10) but not all cases (12). Yadin and colleagues (9) observed deficits at 12 to 14 weeks of age on a simultaneous discrimination task in rats given preweanling MTX treatment but only when it was given in combination with cranial radiotherapy. Winocur and colleagues (42) found no effect on discrimination learning using a black–white discrimination task following treatment of MTX and 5-fluorouracil in adult mice. In contrast, these researchers (43) found impairment on the same discrimination task following identical treatment. The conditional discrimination procedure used in the present study involved an additional requirement by including a hippocampus-sensitive discrimination reversal. Previous studies in the area of developmental toxicology suggest that the reversal component may be more sensitive to disruption following toxicant exposure compared with the initial discrimination learning (44). Impairment exhibited by the higher combination group during the reversal phase is potentially related to the hippocampus-dependent deficits displayed by this treatment group on the novel objection recognition task. Therefore, the current findings fit into the growing body of literature surrounding chemotherapy-induced cognitive deficits, which suggests that tasks dependent on the hippocampus or frontal lobes are more sensitive to chemotherapeutics compared with tasks dependent on subcortical structures such as the caudate nucleus and related striatal structures (42, 43). Although the current findings are more consistent with hippocampus sensitivity, results from this model do not exclude a role for the frontal lobes in chemotherapy-induced deficits; however, additional behavioral tasks would need to be examined to ascertain this relationship. Further research examining differential vulnerability to the neurotoxic effects of chemotherapeutics among brain regions is warranted.

It is important to study the effects of chemotherapy in developing rodent brains, as toxins or stress during early development can have more severe long-term effects than identical damage in the mature adult rodent brain (45, 46). Li and colleagues (8) examined the effects of both acute and chronic MTX treatment using male rats, beginning at PND 15. Their findings are in contrast to the current study in that they observed impairment on an object placement test but not on an object recognition task. However, several differences separate these 2 studies, including differences of species and of dosing regimen. Li and colleagues (8) treated rats with 1 mg/kg MTX, whereas the current study treated mice with either 1 or 2.
mg/kg MTX, and significant deficits were only observed with the higher dose in the current study. It is conceivable that the type of chemotherapy-induced cognitive impairment is dependent on the timing and length of treatment delivery, which could be further affected by strain and sex. Additional studies using preclinical models of childhood chemotherapy treatment are needed to sort through these important variables.

The occurrence of only mild deficits on the conditional discrimination procedure presented here has important clinical implications for rehabilitation of cancer survivors experiencing chemotherapy-induced learning deficits as well because the present results suggest that impairment may be ameliorated through extensive training or practice. This idea is consistent with findings from Boyette-Davis and Fuchs (47), in which rats underwent a paclitaxel treatment regimen that produced mechanical sensitivity but failed to produce impairment on the 5-choice serial reaction time task. Similar to the conditional discrimination procedure used here, this assay included an extensive training phase requiring rodents to reach a 70% criterion before beginning the testing phase. Massed practice and repetition are also central components of a cognitive remediation program proposed for childhood cancer survivors experiencing difficulty in school, which includes techniques derived from brain injury rehabilitation, special education, and clinical psychology (48–50). The beneficial outcome of practice effects is comparable with the concept of overlearning presented by Reid (51), in which rats that received 150 overtraining trials past a criterion level required substantially fewer trials to reach criterion on a new discrimination condition compared with rats trained only to criterion on the initial discrimination.

A notable observation for the current study is the lack of data supporting synergistic toxicity in any measure for the 2 chemotherapeutic agents. However, there is not an a priori reason why MTX and Ara-C should be synergistic or additive for the behavioral endpoints chosen. It is possible that the combinations examined may be subtractive and the administration of one agent diminishes the toxicity of the other (52). Lower doses of MTX in combination with 5-fluorouracil produced greater cognitive deficits than higher doses of MTX in combination with 5-fluorouracil in adult mice (18, 41). Future studies should expand on our assessment through more quantitative methods to further classify these drug interactions. Additional testing of time points (earlier or later) may reveal increased deficits for the combinations compared with single agents.

The experiments described herein represent the foundation for a novel mouse model in the area of cognitive late effects in childhood cancer survivors, and these findings support further investigation. One limitation of the present study is that healthy naïve mice were treated with chemotherapeutic agents, as opposed to mice bearing an ALL-related cancer. Although this is a logical next step, only a couple of laboratories have been able to inject xenografts from patients with ALL into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice to attempt to model ALL with any success (see refs. 53, 54 for review of humanized mouse models). However, the validity of using healthy rodents is supported by the finding that tumor presence does not potentiate an MTX-induced decrease of cell proliferation in the hippocampus (16). In addition, although a number of studies have shown central nervous system toxicity and behavioral deficits for systemic MTX (15) and Ara-C (20) in adult rodents, intrathecal administration would strengthen clinical relevance. Future studies using the present model should assess later time points to clarify whether the deficits observed represent subacute toxicity that would subsequently resolve or yield true persistent deficits, although different learning tasks more amenable to repeated testing than those used in the current study would be required to follow the trajectory of the deficits into adulthood. The present findings do not address mechanisms of neurotoxicity, although such data have been collected in adult rodents (see ref. 7 for review); consequently, future research involving young rodents is warranted.

In summary, the present study shows that early exposure to the chemotherapeutics MTX and Ara-C, presented alone or in combination, can disrupt acquisition and retention of a learned response as well as short-term recognition memory tested once 19 days after treatment. The occurrence of decreased response accuracy following a one-session rapid training in only one chemotherapy treatment group suggests that extensive training or practice may help to ameliorate learning impairment. These findings have clinical implications to support the use of cognitive remediation and similar techniques in school-aged children experiencing cognitive late effects from chemotherapy treatment. The preclinical model presented within a developmental framework is unique, and its continued use is supported by clinically relevant findings. However, the current study also highlights the need for further expansion in the area of preclinical models for the emergent effects of childhood chemotherapeutic treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: E.B. Bisen-Hersh, P.N. Hineline, E.A. Walker
Development of methodology: E.B. Bisen-Hersh, P.N. Hineline, E.A. Walker
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.A. Walker
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.B. Bisen-Hersh
Writing, review, and/or revision of the manuscript: E.B. Bisen-Hersh, P.N. Hineline, E.A. Walker
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.B. Bisen-Hersh
Study supervision: E.A. Walker

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References
24. Barrett JE, Vanover KE. Assessment of learning and memory using the autoshaping of operant responding in mice. Curr Protoc Neurosci Hematol 2004;Chapter 8:Unit 8.5F.


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Emily B. Bisen-Hersh, Philip N. Hineline and Ellen A. Walker

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