ABSTRACT

Central nervous system prophyactic therapy used in the treatment of acute lymphoblastic leukemia can reduce intelligence quotient scores and impair memory and attention in children. Cranial irradiation, intrathecal methotrexate, and steroids are commonly utilized in acute lymphoblastic leukemia therapy. How they induce neurotoxicity is unknown. This study employs an animal model to explore the induction of neurotoxicity. Male and female Sprague-Dawley rats at 17 and 18 days of age were administered 18 mg/kg prednisolone, 2 mg/kg methotrexate, and 1000 cGy cranial irradiation. Another 18-day-old group was administered 1000 cGy cranial irradiation but no drugs. Matching controls received saline and/or a sham exposure to radiation. All animals at 6 weeks and 4 months of age were tested for alterations in spontaneous behavior. A computer pattern recognition system automatically recorded and classified individual behavioral acts displayed during exploration of a novel environment. Measures of behavioral initiations, total time, and time structure were used to compare treated and control animals. A permanent sex-specific behavioral change in the time structure of behavior was induced by the prednisolone, methotrexate, and radiation treatment but not by radiation alone. Unlike hyperactivity, the effect consisted of abnormal clustering and dispersion of acts in a pattern indicative of disrupted development of sexually dimorphic behavior. This study demonstrates the feasibility of an animal model delineating the agent/agents responsible for the neurotoxicity of central nervous system prophylactic therapy.

INTRODUCTION

Children with ALL routinely receive CNS prophylactic therapy to prevent CNS relapse and in turn systemic relapse of the disease. While CNS treatment has improved long term survival rates, its side effects have become an important consideration. Neurotoxicity is paramount among these concerns. Children given CNS prophylactic therapy have significant reductions in overall intelligence quotient scores, as well as impaired verbal and visual-spatial memory abilities, attention, organization, and motor output (1-6). Occurrence of these effects has been shown to depend upon gender (6-9), and often impairment is sufficient to warrant supplemental special education or even education outside the regular classroom setting. The seriousness of these side effects requires consideration of toxicity as well as efficacy. Therefore, avoiding cognitive impairment while maintaining therapeutic efficacy of CNS prophylaxis has become an aim of current and future leukemia treatment protocols.

Cranial irradiation and intrathecal methotrexate administration are the two treatments most often involved in CNS therapy (10). In addition, daily doses of steroids are administered during remission induction and sometimes during CNS prophylaxis (11). All three have neurotoxic potential, but some suspect cranial irradiation plays the major role in cognitive impairment (2). Indeed, a preponderence of studies have shown that patients receiving radiation were more impaired than those not exposed (12). However, omitting cranial irradiation from a treatment protocol and using only high doses of methotrexate produced cognitive decrements similar to those seen when cranial irradiation was combined with standard doses of methotrexate (5). Thus, omitting radiation does not necessarily mitigate neurotoxicity. Nonetheless, in an effort to reduce neurotoxicity, clinicians increasingly are exploring CNS treatments that exclude cranial irradiation and/or intrathecal drugs (13-15), without adequate information on neurotoxic mechanisms. In contrast, the neurotoxic potential of steroids is being ignored, despite clear sex differences in the occurrence of cognitive impairment. CNS treatment protocols, therefore, are being manipulated in ways that might risk adverse consequences whose extent will not be known for years.

Children with ALL receive multiple courses of systemic as well as CNS treatment and, thus, isolating a single agent or a combination of agents responsible for neurotoxicity is extremely difficult. The CNS prophylactic therapy is so crucial to event-free survival that clinical protocols cannot be systematically manipulated to determine neurotoxic mechanisms. Therefore, what causes cognitive impairment is still an open question. An animal model is examined in this study to compare the effects of a combination of therapeutically relevant levels of cranial irradiation, methotrexate, and prednisolone with those of cranial irradiation alone, the single agent most often suspected as being neurotoxic. Behavioral response is measured using new computer pattern recognition technology, which is designed for dose-response determinations (16), objective identification of rat behaviors (17), and analyses sensitive to CNS malfunction (18-21). No study of an animal model has provided such extensive screening for the neurotoxic potential of these agents involved in CNS treatments.

MATERIALS AND METHODS

Animals

The Sprague-Dawley male and female rats examined here were those described in the growth study reported in the accompanying paper (22). Again, the four treatment groups studied were the following: RPM, pups received on days 17 and 18 a combined therapy involving 1000 cGy cranial radiation, 18 mg/kg prednisolone, and 2 mg/kg methotrexate; RPM-C, this control group received sterile saline on days 17 and 18 and a sham exposure to radiation on day 18; XRT, pups received 1000 cGy cranial radiation on day 18; and XRT-C, this control group for the XRT animals received only a sham exposure to radiation on day 18.
Analysis of Behavior

Behavior was analyzed in the rats at 6 weeks of age and again at 4 months of age. The tests were conducted during the diurnal period between 9:00 a.m. and 1:00 p.m. each day. A pair of rats, one experimental and one matched control, were placed simultaneously into a Plexiglas box and tested during the first 15 min of exploration of the novel environment. The experimental and control rats were separated by a clear partition with small holes, which allowed the animals to see and smell each other during the test. Two video cameras taking one frame/s were used to monitor their spontaneous behavior. The video signals were transferred to a MICRO VAX I and a VAX 11/750 for pattern analysis and behavioral classification of the data. The specific behaviors identified by the computer consisted of five major body positions, stand, sit, rear, walk, and lie down, and eight modifiers, blank (no recognized activity), groom, head turn, turn, look, smell, sniff, and wash face. Details of the test environment, the test procedure, and the computer pattern recognition system have been described (17).

Three measures of spontaneous behavior were taken, measures known to contain independent information about CNS control of motor output (18, 20), as follows.

Calculation of Behavioral Initiations. The number of frames in which a specific behavioral act began was totaled for the 15-min observation period for each rat. The mean number of initiations was determined for each act for the control and experimental rats. Statistical significance was determined using a t test, and a P < 0.05 was required for a change to be labeled as significant.

Calculation of Behavioral Total Time. The number of frames that a behavior continued, including the frame it was initiated, was totaled for the 15-min observation period. The mean total time for each act in control and experimental rats was determined, and statistical significance was assessed using the t test. Again, a P < 0.05 was required for a change to be considered significant.

Calculation of Time Distribution and Time Sequence. The time distribution and time sequence of behavioral acts was calculated using equations for \( K(t) \), as previously described (18, 20). When the rats were 6 weeks of age, the function \( K(t) \) was computed for four data sets: (a) 15 pairs of RPM-treated and RPM-C males, (b) 18 pairs of RPM-treated and RPM-C females, (c) 20 pairs of XRT-treated and XRT-C males, and (d) 20 pairs of XRT-treated and XRT-C females. At 4 months of age, the data from the same groups were analyzed, but by then the number of male XRT and control pairs was reduced to 17, due to prior sacrifice for pathological examination. Each pair was composed of one control and one experimental animal. For each behavioral time distribution or time sequence, a \( \Delta K(t) \) [the difference between \( K(t) \) for the exposed animals and matching controls of a particular data set] was calculated for eight time points (2, 5, 10, 20, 30, 45, 100, and 200 s). The bootstrap technique was used to estimate the SD in this measure (18). The ad hoc criteria for the significance of an observed change between treated rats and matching controls have been described (18). Whenever any time distribution or sequence involved a behavioral act which had an average number of initiations (per animal) less than 10 in either the control or experimental group, the \( K(t) \) values were not determined.

The \( K \) function was calculated for specific behavioral acts (e.g., sit, rear) or sequences of specific behavioral acts (sit . . . rear), as described by Mullenix and Kernan (20). In addition, \( K \) functions were determined for combined acts (e.g., attention or attention/groom) and sequences of combined acts (e.g., attention . . . explore or attention/explore . . . groom/attention), as described by Kernan and co-workers (18). Interpretation of the behavioral effects of CNS prophylactics was facilitated by our previous studies of positive and negative controls (18–20). The false positive error rate that can be expected with the computer pattern recognition system is approximately 10% in the specific act analysis and 8% in the combined act analysis (19). Therefore, a percentage change in any treatment group was not considered significant unless these false positive rates were exceeded.

RESULTS

The time structure analysis of combined behavioral acts was found to be the most effective measure to delineate XRT from RPM effects. It revealed that the RPM treatment induced a permanent sex-specific malfunction and that XRT alone did not (Table 1). The RPM-treated males were particularly affected; 48% (12 of 25) of their combined acts and sequences were altered significantly at 6 weeks of age. At 4 months of age, significant change in combined acts and sequences (22%, 8 of 36) was detected again in the same group of animals. In contrast, the percentage of change in XRT animals and the RPM females was no greater at either age than that expected by chance (0–8%) in the combined act analysis (19).

Of the combined acts affected in the male RPM rats, some had \( K \) functions that were increased, indicating increased clustering in time, and some had \( K \) functions that were reduced, indicating increased dispersion in time (Table 1). Fig. 1A demonstrates significant clustering of the attention/groom behavior; during the 15-min observation period there were more initiations of this behavior separated by 2–45-s intervals in RPM males compared to controls. Fig. 1B demonstrates significant dispersion of the behavioral sequence attention/explore . . . groom/explore; during the 15-min observation period there were fewer initiations of this sequence separated by 2–45-s intervals in RPM males compared to controls.

The particular combined acts and sequences affected at either age are listed in Table 2. Two combined acts and three of their sequences appeared to be consistent markers of RPM treatment in males, because they were changed the same way (clustered or dispersed) at both ages. Furthermore, whenever behavioral time structure was altered, it changed in a pattern to become more like the time structure in control females. This shift could be seen in all combined acts (a total of 11) for which control female \( K \) values were available for comparison. The feminization of male behavioral time structure by the RPM treatment is illustrated for three combined acts in Fig. 2.

In addition to the time structure analysis of combined acts, the measures of combined act initiations and total times revealed more of the treatment effect. A summation of significant changes using all three measures is shown in Table 3. The RPM males clearly had the largest total number of significant

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effects of RPM and XRT treatments on the time structure of combined acts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male rats exposed on days 17 and 18 to 1000 cGy, 18 mg/kg prednisolone, and 2 mg/kg methotrexate (RPM) had significant changes in behavioral time structure at 6 weeks and 4 months of age. RPM-exposed females were not affected. Rats exposed on day 18 to 1000 cGy (XRT) did not have significant changes in behavioral time structure.</td>
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</table>

<table>
<thead>
<tr>
<th>XRT</th>
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<th></th>
<th>Total changed (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>K functions analyzed</td>
<td>K functions clustered</td>
<td>K functions dispersed</td>
<td>K functions changed</td>
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<tr>
<td>Males</td>
<td>6 wk</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4 mo</td>
<td>25</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Females</td>
<td>6 wk</td>
<td>16</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4 mo</td>
<td>16</td>
<td>1</td>
<td>0</td>
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<tr>
<td>RPM</td>
<td></td>
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<td>Total changed (%)</td>
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<td></td>
<td></td>
<td></td>
<td>48.0</td>
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<tr>
<td>Males</td>
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<td>8</td>
</tr>
<tr>
<td></td>
<td>4 mo</td>
<td>36</td>
<td>5</td>
<td>3</td>
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<tr>
<td>Females</td>
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<td>0</td>
</tr>
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<td></td>
<td>4 mo</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* \( P < 0.0000001. \)

* \( P < 0.00637. \)
NEUROTOXICITY OF CNS THERAPY FOR ALL

**Fig. 1.** Behavioral time structure was altered in RPM male rats (●) compared to controls (○). In A, K functions are the time distributions of the combined act attention groom in 6-week-old rats. In B, K functions are the time sequence of attention/explore . . . groom/explore in 6-week-old rats. Error bars, SD.

**Table 2 Combined acts and sequences in RPM males that were clustered or dispersed with respect to time**

The age/ages at which each act or sequence was affected is shown. GR, groom; EXP, explore; ATT, attention.

<table>
<thead>
<tr>
<th>Combined acts affected in RPM males</th>
<th>Age affected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clustered</td>
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<tr>
<td>Gr/Exp</td>
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</tr>
<tr>
<td>Gr/Att</td>
<td></td>
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<tr>
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</tr>
<tr>
<td>Gr/Exp . . . Gr</td>
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</tr>
<tr>
<td>Dispersed</td>
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</tr>
<tr>
<td>Exp . . . Gr/Exp</td>
<td>3</td>
</tr>
<tr>
<td>Exp . . . Att/Gr</td>
<td>1</td>
</tr>
<tr>
<td>Gr/Exp . . . Exp</td>
<td>1</td>
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<tr>
<td>Gr/Exp . . . Att</td>
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</tr>
<tr>
<td>Att . . . Att/Gr</td>
<td>1</td>
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<td>Att/Gr . . . Att/Exp</td>
<td>1</td>
</tr>
<tr>
<td>Att/Exp . . . Gr/Exp</td>
<td>1</td>
</tr>
<tr>
<td>Exp . . . Att</td>
<td>2</td>
</tr>
<tr>
<td>Att . . . Exp</td>
<td>2</td>
</tr>
</tbody>
</table>

* I = 6 weeks; 2 = 4 months; 3 = 6 weeks and 4 months.

changes. The XRT males had the next highest total, only slightly more than those observed in RPM or XRT females. While the number of behaviors altered in time structure decreased with age, the number of behaviors altered in initiations and total time increased with age (Table 3). Two of the combined acts altered in time structure in the 4-month-old RPM males were the same two which were altered in initiations and total time. RPM males initiated groom/attention acts an average of $43.0 \pm 3.8$ (±SE) times, while this measure for the matching controls was $60.3 \pm 4.7$ times. Also, the average total time of this combined behavior was shorter in RPM males ($86.4 \pm 9.2$ s) compared to controls ($136.7 \pm 16.5$ s). Average initiations of the combined act attention/explore increased in RPM males ($126.7 \pm 5.6$) over controls ($108.3 \pm 4.9$), as did the average total time of this combined act (RPM males = $183.8 \pm 8.8$ s; controls = $154.1 \pm 7.9$ s).

One consistent factor pervaded RPM and XRT effects using the initiation and total time measures of specific acts; of the 13 initiation and total time changes observed, 8 changes involved the act of sitting, and the change was always a decrease in its initiations and shortening of its total time. For example, 4-month-old RPM males initiated sitting less often ($40.9 \pm 3.5$ times) than respective controls ($53.9 \pm 3.7$ times), and compared to controls ($189.6 \pm 22.8$ s) they shortened the duration of sitting ($121.9 \pm 15.4$ s). This effect on sitting behavior occurred in both sexes and regardless of treatment (XRT and RPM).

**DISCUSSION**

A combination of cranial irradiation, prednisolone, and methotrexate was found to induce permanent neurotoxicity, but not...
hyperactivity, in young male rats. Cranial irradiation alone, in contrast, had relatively little effect. The XRT-treated male rats had minor changes in spontaneous behavior, enough to suspect that doses much higher than 1000 cGy might indeed produce substantial CNS effects. Within the dose range relevant to CNS prophylactic therapy, however, the results indicate that methotrexate and prednisolone may play the more dominant role in this sex-dependent neurotoxicity. A careful dose-response curve is needed for each agent individually before its relative contribution to neurotoxicity can be evaluated.

Mental retardation and gross hyperactivity are not reported in children given CNS prophylactic treatment. Rather, these children are best characterized as learning disabled, precluding normal academic and social development. A valid animal model to study such cognitive dysfunction requires behavioral measures with unusual sensitivity. To accommodate this subtlety in animals, measures of spontaneous behavior were adopted, instead of the usual tests of one conditioned response as employed in earlier studies (23). The idea of using measures of spontaneous behavior to detect cognitive deficits gained impetus when neurophysiological evidence indicated the CNS, independent of sensory feedback, was the source of temporal regulation of behavior (24). Lashley (25) proposed that the CNS had high speed autonomous oscillators that regulated temporal structure of rhythmical motor sequences. The frequency and temporal course of spontaneous stereotyped behavior were studied in mentally retarded individuals (26, 27) and autistic children (28), and brain damage from different situations of perinatal distress was compared using measures of spontaneous sucking rhythms in infants (29). Moreover, spontaneous behaviors, e.g., body rocking, head nodding, hand waving, and head banging, were analyzed in retarded individuals, and it was found that the frequency of these spontaneous behaviors was negatively correlated with measured intelligence (26). Problems of measurement limited analyses of spontaneous behavior in humans (26, 27), but such problems have been overcome by the technology applied in this animal study (16–21). Consequently, the full repertoire of spontaneous behaviors is now considered, and behavioral initiations, duration, and temporal structure are analyzed to effectively screen for overall CNS malfunction.

On the basis of simple movements discernible to the eye, RPM males and controls could not be distinguished. With the exception of sitting, few single acts changed in initiations or total time. Visible cues of hyperactivity, i.e., increased initiations of walking and turning (16, 30), did not occur. The RPM response could be appreciated as a dramatic effect on behavior only when time structure analyses were applied, and the effects were compared to those induced by other well known CNS-active drugs. For example, the percentage of behavioral time distributions and sequences affected in RPM males (48% at 6 weeks and 22% at 4 months) approximated those observed in rats following acute injections of amphetamine (16) or a prenatal exposure to phenytoin, an exposure associated with permanent mental deficiency (18, 31).

The RPM males shared another notable characteristic with phenytoin-treated animals. They both had a pattern of arrested development of sexually dimorphic behaviors. The RPM males displayed many behavior time structures typical of females, resulting in certain behaviors and sequences inappropriately clustered in time while others were dispersed. The authors have seen (but not discussed) this male/female (or vice versa) conversion in behavioral time structure in other studies whenever exposure occurred during early development (18, 20, 31). Arrested sexual dimorphism in behavior, therefore, may not be unique to the RPM treatment but may result from neurotoxic insults as long as exposure occurs during a critical period in development. Male mice exposed to corticosterone on postnatal days 3–5 display increased adult levels of submissiveness (32). The present findings in RPM rats extend the critical period beyond the first and second postnatal weeks and reveal that individual spontaneous behavior as well as social behavior can be at risk. This male/female conversion does not always appear. Six-week-old male rats administered amphetamine do not display behavioral time structures typical of females; behavioral time structure instead has increased dispersion overall (16). Further evaluation of days 17 and 18 in the male rat is needed to confirm a period critical to the emergence of sexually dimorphic behaviors. Administration of CNS prophylactic therapy in the future may require consideration of such critical periods in the development of sexually dimorphic spontaneous behaviors in children.

Although males displayed the most disrupted behavior in this study, it cannot be assumed that males exclusively are the susceptible sex. On the contrary, in studies of children treated for ALL, cognitive impairment was more prevalent among females (6). A potentially critical variable in this instance again is the age at exposure. Many studies have shown that children given CNS prophylactic therapy at younger ages are at greater risk for long term sequelae (2, 33, 34), but the relationship of age at exposure to sex differences in outcome remains to be explored.

Animals in the present study were exposed at 17 and 18 days of age, a period when some developmental events have been compared with those in humans at birth (23, 35). Had exposure been delayed a few days, different developmental events would be occurring. As indicated by studies of the ontogeny of estrogen, androgen, and progesterin receptors in the rat brain (36–40), a few days can mean a major difference. The brain responds differently to drugs depending upon which hormones are present at the time and upon whether the brain is male or female.
Expression of neurotoxicity would then depend upon the stage of development of the brain-pituitary-gonadal axis at the time of exposure. In primates the orbital cortex matures earlier in males than in females, and such developmental differences are considered the reason why consequences of perinatal injuries appear more frequently in males than females (42). The neurotoxicity in RPM-treated male rats may be related to a similar developmental disparity. In fact, if this study was repeated using an older age at exposure, a different sex-related expression of neurotoxicity might appear.

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An Animal Model to Study Toxicity of Central Nervous System Therapy for Childhood Acute Lymphoblastic Leukemia: Effects on Behavior


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