Long-Term Oral Administration of Memory-Enhancing Doses of Tacrine in Mice: A Study of Potential Toxicity and Side Effects

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Recently, tacrine (1, 2, 3, 4-tetrahydro-9-aminoacridine; THA; TAC) has received international attention as an oral agent capable of relieving some of the cognitive symptoms accompanying Alzheimer's disease (AD). When given acutely and parenterally (by injection), tacrine has also enhanced memory retention in animals and man. This study evaluates the clinical potential of this agent by assessing toxicity and major side effects of a memory-enhancing dose of tacrine in mice. Groups of mice received either tacrine or vehicle (placebo) orally for 4 or 6 months. A lack of toxicity after this prolonged treatment with TAC was indicated by: (a) no significant impairment on a battery of behavioral toxicity tests; (b) improved memory retention; (c) a significant but only slight elevation of ornithine transcarbamylase activity in blood serum; (d) no abnormality as revealed with light microscopy of liver tissue; and (e) no gross organ pathology in visceral organs.

Several types of memory impairment are associated with the aging process in health and disease. These range from the clinically inconsequential mild forgetfulness of healthy old age to the functionally impairing amnesias and dementias of diverse etiologies that more commonly aggress the aged brain. Of these geriatric brain diseases, the most frequently occurring, progressive, and destructive of memory and higher cognitive function is Alzheimer's disease (AD).

Over a decade ago evidence began to suggest that central cholinergic failure might play a major role in the primary (cognitive) symptoms of AD, and, to a certain extent, in the mnemonic deficits observed in the normal aged individual as well. Many recent human and animal studies have contributed to the implication of the central cholinergic system in the maintenance of memory (Bartus et al., 1982). These studies led to the development of an experimental cholinergic pharmacotherapy and to numerous human and animal trials, attempting to improve memory through the administration of acetylcholine precursors, postsynaptic agonists, acetylcholinesterase inhibitors, or combinations thereof (Bartus et al., 1982; Cherkin & Flood, 1985; Davis et al., 1981; Flood et al., 1985; Kaye et al., 1982; Summers et al., 1981).

Within the limited spectrum of cholinergic drugs available for human use, some agents active at the postsynaptic receptor site (arecoline) or at the synapse (physostigmine) have beneficial effects on learning and memory in animal models and humans (Bartus, 1982; Davis et al., 1981). However, their toxic and pharmacologic properties have limited their application in the treatment of memory impairment (Rosenberg et al., 1983).

Among the few anticholinesterases presently available for clinical use, tacrine (1,2,3,4-tetrahydro-9-aminoacridine; TAC) was recently suggested as an agent with potential for memory enhancement (Summers et al., 1981) and was tried parenterally with success in mice (Flood et al., 1985) as well as with some success in a few patients with Alzheimer's disease (Kaye et al., 1982; Summers et al., 1981). Summers et al. (1986) focused international attention on TAC by reporting significant and persistent cognitive improvement and enhanced functional performance in 12 AD patients to whom the drug was orally administered for periods lasting up to several months without notable adverse effects. No other drug given to AD patients in a controlled trial has yet to produce cognitive effects as robust as those reported by this group. For many years, parenterally administered TAC was used in man to treat acute anticholinergic syndrome, phencyclidine intoxication and myasthenia gravis (Summers et al., 1980). TAC has several pharmacologic properties that recommend it as an agent with the potential for oral administration. These are a half-life of approximately 5 to 8 hr, infrequent peripheral side effects, and low CNS toxicity with short-term administration. However, definition of its long-term effects remains incomplete.

The purpose of this study in mice was to determine if long-term oral ingestion of TAC results in hepatotoxicity, tolerance, or behavioral toxicity at a dose level found to improve memory when given over 2 weeks (Flood et al., 1984). Chronic oral administration was chosen to provide information that would assist in the development of a practical pharmacotherapy of age-related amnestic disorders and cognitive decline in man.

MATERIALS AND METHODS

Eighty C57BL/6Nia male mice obtained at 8 weeks of
age from the National Cancer Institute were divided equally into four groups: two control groups (4-month vehicle, 6-month vehicle) and two experimental groups (4-month TAC, 6-month TAC). TAC was obtained from Aldrich Chemical Company (Milwaukee, WI). The 2-month mean body weight and standard deviation was 22.1 ± 1.3 grams. The mice were housed individually and provided with drinking fluid that contained 0.02% sodium saccharin and 0.0015% methyl salicylate (oil of wintergreen) in distilled water. This vehicle masked any unpleasant taste when TAC was added. During the first week, all mice were provided with the vehicle only. During the second week, TAC 0.2 mg/ml was added to the vehicle of the two experimental groups while the others continued receiving vehicle alone. Fresh solutions were provided at 2- to 3-day intervals. Mice were maintained on these regimes for either 4 or 6 months, at which time the mice were subjected to a battery of behavioral tests. After these tests, the mice (8 and 10 months of age) were anesthetized with methoxyflurane for approximately 30 sec. Blood samples were obtained by ventricular heart puncture and allowed to clot at room temperature. The serum was used for enzymatic analysis. Liver tissue was removed immediately after sacrifice for histological studies.

Biochemical toxicity studies. — Ten mice from each group were randomly selected for biochemical and histological studies. Serum ornithine transcarbamylase (OTC) activity, a specific index of hepatocellular integrity (Jones et al., 1961; Vassef, 1978), was monitored by a modification of the established method of Jones et al. (1961). The assay is based upon the conversion of citrulline to ammonia. Citrulline in the presence of arsenate and OTC is converted to carbamyl arsenate, which decomposes spontaneously with ammonia as one of the end products. The formation of ammonia is the measure of OTC activity. In practice, 150 ul of blood serum were added to 1000 ul of a solution of 1.0M sodium arsenate (pH = 6.9) and 0.025M citrulline. This mixture was incubated in a stoppered tube for 24 hr at 37 °C. (The long incubation period reflects the low OTC level in normal mammalian blood serum.) Incubation was terminated by adding enough 1.0M sulfosalicylic acid to achieve a final concentration of 0.25M. The denatured proteins in the mixture were precipitated by centrifugation in an Eppendorf microfuge, and 125 ul of the supernatant were assayed for ammonia (Vassef, 1978). Suitable blanks and a standard curve for the ammonia assay were included with each assay.

Histological toxicity studies. — In a preliminary effort to determine if long-term ingestion of TAC induced histopathological changes in liver tissue, three livers from the control and three from the 6-month TAC group were randomly selected and prepared according to established techniques for histological examination (Mallory, 1938). Liver tissues of experimental and control mice were fixed in Bouin’s solution, dehydrated, cleared, and infiltrated with hot paraflast. Sections were cut in 6 micron slices, stained with hematoxylin eosin, and examined histologically under low- and high-power light microscopy, using blind-coded specimens to avoid experimenter bias.

Behavioral toxicity tests. — All mice were tested on a battery of tasks designed to determine their general state of health, which included (a) activity wheel (revolution/20 min test) and rotorod (sec on wheel) to measure motor activity, coordination, and endurance (Dunham & Miya, 1957); (b) tight rope (sec on rope) and grip strength test (grams displaced on strain gauge) to determine muscle tone and strength (Cabe et al., 1978; Irwin, 1968); and (c) negative geotaxic reflex on an inclined plane (degrees vertical movement) (Cabe, 1978). Rectal temperature also was recorded.

Drug toxicity in rodents is frequently associated with a decrease in activity and a lack of motor coordination and strength. Healthy mice given access to an activity wheel will readily run in it, whereas mice that are ill usually turn the wheel less than 100 times during a 20-min test. Because mice must learn to operate the wheel, they were given three 20-min practice sessions prior to measuring activity. The rotorod is a large drum that turns a little faster every second eventually reaching 80 rpm. The object of the test is to determine how well a mouse is able to keep pace with the turning drum. A mouse is placed on the drum, and the rotation starts at 1 rpm. As the rpm is increased, it becomes more difficult for a mouse to move fast enough to avoid falling onto a shock grid below. This task also requires learning that falling results in a 2-sec 0.4 ma footshock. Thus, mice were given two training sessions, two per day for 2 days, prior to measuring for how many revolutions they could remain on the rotorod. The test score was the average of two test runs on the third day. The task requires coordination and endurance. The rotorod differs from the activity wheel in that the rotorod is forced activity, whereas the activity wheel measures spontaneous activity. Drug toxicity would be expected to reduce muscle strength; the tight rope and grip strength tests measured muscle strength primarily. The tight rope test consists of lowering a mouse by the tail near a 0.3 cm cord. Once the mouse has grabbed the rope with its forepaws, the tail is released. Healthy mice are able either to hold onto the rope or swing their body around and grab the rope with the back feet as well. If the mouse does not fall from the rope in 30 sec, it is removed. The mice learn quickly to hold on to the rope. Three tests were run over a 10-min period; an average of the last two tests was used as the mouse’s test score. The grip strength apparatus consists of a strain gauge with a ring attached to it. The mouse is lowered by the tail until it grabs the ring. Once the mouse grabs the ring, the experimenter pulls slowly and as constantly as possible until the mouse releases its grip. The strain gauge automatically records grams displaced. The tests were run with 10 min interbetween tests. The average grams displaced over the two tests was used as the test score.

Mice exhibit a negative geotaxis when placed on a 60-degree inclined plane. The inclined plane consists of a fine wire mesh tightly stretched over a frame. A healthy mouse walks up the inclined plane at about 90 degrees from the base of the plane; if the mouse is ill or intoxicated, it usually walks along a path with an incline that is less than 30 degrees. The results of two test runs over a 10-min period were averaged. Some drugs affect body temperature; thus, rectal temperature was taken.
Memory retention test. — At approximately 6 and 8 months of age, when the mice had received either 4 or 6 months of TAC treatment, respectively, all mice were trained in a T-maze to avoid footshock (Flood et al., 1975). Mice received four training trials. One week later, retention was measured by resuming training until a mouse made five avoidance responses in six consecutive trials (criterion) in one session. The two measures of retention were the mean number of trials to first avoidance response and to reach criterion. Those mice making their first avoidance in three trials or less were classed as remembering the original training. This criterion was adopted because it provided optimal separation between the retention test scores of naive mice (those without prior T-maze training) and well-trained mice (Flood et al., 1975). The recall score is the percentage of mice in a group that made their first avoidance on trials 1, 2 or 3.

RESULTS

Fluid consumption. — In this study, fluid consumption decreased significantly after the first 4 months of treatment (Table 1). The effective mean dose of TAC ingested during the 3 weeks before testing decreased from 41.33 mg/kg/day after 4 months to 28.78 mg/kg/day after 6 months. A similar decrease in fluid consumption occurred in the control group. The 31% decrease in dose of TAC ingested per unit body weight resulted because of an 18.4% decrease in fluid intake and a 12.6% increase in body weight.

Biochemical toxicity. — It is estimated that the mice consumed a mean total dose of 4,542 mg/kg of TAC over 4 months and 6,288 mg/kg over 6 months. The effect of the TAC ingested upon liver function as measured by OTC serum levels is shown in Table 1. After 4 months of treatment, the average OTC activity in serum was 65% above that of controls. After 6 months of ingesting TAC, OTC activity in the TAC group was about the same as after 4 months. The OTC levels (NH3/24 hr/ml serum) were analyzed by a two-way analysis of variance (ANOVA) for treatment duration by drug treatment, $F(3,36) = 4.38$, $p < .01$. The main effect of treatment duration was not significant, but drug treatment was $F(1,36) = 12.20$, $p < .005$, indicating that TAC-treated mice had higher OTC levels on the average than vehicle-treated mice, whether they were treated over a 4- or 6-month period. The distribution of OTC levels in treated and untreated mice is shown in Figure 1.

Histology. — Representative histological findings are shown in Figure 2. In all, more than 300 sections were examined, and in none was there indication of acute or chronic toxic or inflammatory changes (e.g., lobular necrosis, leucocytic infiltrates, deposits, hepatocyte abnormalities, portal fibrosis) after either 4 or 6 months of treatment with TAC or vehicle. No gross pathology was found on inspection of other visceral organs in experimental mice.

Table 1. Effect of Prolonged Oral TAC Intake on Body Weight, Fluid Consumption, and Ornithine Transcarbamylase

<table>
<thead>
<tr>
<th>Measured parameters</th>
<th>4-month treatment</th>
<th>6-month treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle control</td>
<td>Tacrine treated</td>
</tr>
<tr>
<td>Age at time of testing (months)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (gm) ($M \pm SEM$)</td>
<td>25.1 ± 0.3</td>
<td>25.5 ± 0.3</td>
</tr>
<tr>
<td>Fluid intake (ml/day) ($M \pm SEM$)</td>
<td>5.85 ± 0.13</td>
<td>5.27 ± 0.13</td>
</tr>
<tr>
<td>Daily TAC intake (mg/kg/day)$^a$</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>Ornithine transcarbamylase$^b$ ($M \pm SEM$)</td>
<td>100.3 ± 7.4</td>
<td>165.2 ± 14.2</td>
</tr>
</tbody>
</table>

$^a$Both weight and fluid intake differed significantly ($p < .001$) between the 6- and 8-month old mice, but differences between the control and TAC groups were not significant.

$^b$Averaged for 3-week period prior to testing and sacrifice based on ml/day of fluid consumed.

Serum ornithine transcarbamylase levels were significantly elevated above controls in tacrine-treated mice ($p < .01$) after 4 months of treatment. Time and the interaction of time and drug were not significantly interrelated to the enzyme levels as tested by a two-way analysis of variance (ANOVA).
Behavioral toxicity and assessment of memory retention.

Table 2 summarizes the results of the behavioral toxicity test for mice treated 4 or 6 months with tacrine or vehicle. No significant differences in performance on the tight rope, inclined plane, grip strength, rotorod, or activity wheel tests were found. Table 2 also contains the results of retention testing. Mice treated with TAC for 4 or 6 months showed significantly better retention than vehicle-treated mice ($p < .001$, Student's $t$ test using means to criterion). Based on trials to first avoidance, TAC mice treated for 4 months had a recall score of 70% versus 20% for their control, and mice treated for 6 months had a recall score of 75% versus 20% for their control. This enhancement of retention test performance apparently was obtained without enhancement of acquisition (i.e., learning), as no significant differences in latency measures during training existed between tacrine- and vehicle-treated mice.

DISCUSSION

TAC ingested by mice at a memory-enhancing dose produced no behavioral toxic effects; although OTC levels were significantly elevated, no toxicity was detectable by histological examination of liver tissue. Following earlier studies in which TAC was administered for only 2 weeks to mice (Flood et al., 1984), the present long-term study confirms that TAC produced significant improvement in memory retention over a 4- and 6-month period or approximately 75% of their lifetime. These results suggest that long-term ingestion of TAC does not result in pharmacologic tolerance. An agent that improves memory initially but after several months of administration produces tolerance would not be expected to improve memory retention without progressive dosage increases.

The lack of evidence suggesting drug toxicity on liver or on behavior is noteworthy; however, it does not rule out possible histological long-term effects of drug exposure on bone marrow, kidney, spleen, or heart. No macrophraghia was evident in these organs. The modest hepatocyte enzyme (OTC) serum activity elevation in the TAC-treated mice, occurring in the absence of any liver histopathology, suggests hepatocyte membrane permeability changes rather than a cellular necrotic process (Simmerman & Seeff, 1970). Furthermore, not all of the mice exhibited OTC elevation in either the 4- or 6-month treated groups (Figure 1), underscoring the idiosyncratic nature of the enzyme elevations. Several agents in current clinical use, for example clofibrate (Schwandt et al., 1978) and chenodeoxycholic acid (Bateson et al., 1977; Fromm et al., 1975; Tan & Warren, 1982), can produce in humans transient changes in the level of hepatic enzymes in blood. In 8-month-old mice, the normal level of serum OTC ranged from 36 to 178

<table>
<thead>
<tr>
<th>Test</th>
<th>Vehicle control</th>
<th>Tacrine treated</th>
<th>$p$</th>
<th>Vehicle control</th>
<th>Tacrine treated</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at time of testing (months)</td>
<td>6</td>
<td>6</td>
<td></td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Tight rope (sec on rope)</td>
<td>14 ± 2.1</td>
<td>9 ± 2.1</td>
<td>NS</td>
<td>15 ± 3.7</td>
<td>21 ± 4.1</td>
<td>NS</td>
</tr>
<tr>
<td>Inclined plane (degrees vertical)</td>
<td>87 ± 0.9</td>
<td>87 ± 0.4</td>
<td>NS</td>
<td>88 ± 0.8</td>
<td>87 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Grip strength (gm displaced)</td>
<td>184 ± 8</td>
<td>176 ± 10</td>
<td>NS</td>
<td>208 ± 10</td>
<td>199 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Rotorod (sec on wheel)</td>
<td>217 ± 13</td>
<td>241 ± 16</td>
<td>NS</td>
<td>248 ± 18</td>
<td>239 ± 18</td>
<td>NS</td>
</tr>
<tr>
<td>Activity wheel (revolutions)</td>
<td>419 ± 47</td>
<td>381 ± 44</td>
<td>NS</td>
<td>484 ± 42</td>
<td>442 ± 43</td>
<td>NS</td>
</tr>
<tr>
<td>T-Maze retention</td>
<td>9.2 ± 0.3</td>
<td>7.0 ± 0.3</td>
<td>.001</td>
<td>9.2 ± 0.2</td>
<td>7.2 ± 0.3</td>
<td>.001</td>
</tr>
<tr>
<td>($M$ trials to criterion)</td>
<td>20</td>
<td>70</td>
<td></td>
<td>20</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

Note. Means and standard errors of the means are given for various tests of behavioral toxicity as well as tacrine's effect of retention for footshock avoidance conditioning in a T-maze. $n = 20$ mice in each group. All statistical comparisons were done using Student's $t$ test, except for recall score, which was evaluated by Fisher's Exact Probability Test.
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nmolues NH4/24 hr/ml serum. Thus, the serum OTC levels of all but 40% of the mice treated with TAC for three-quarters of their lifetime fell within the upper limits of the normal range (Figure 1). No hepatic enzyme elevations were observed when up to 90 mg of TAC per day were administered for 2 weeks to humans with tardive dyskinesia (Ingram & Newgreen, 1983).

In the current study, the water consumption of mice decreased, and their body weight increased from 6 to 8 months of age. This accounts for the differential TAC ingestion between the 4- and 6-month groups. The control groups reduced their fluid intake and increased their body weight in a similar manner with age. Thus, the decrease in TAC ingested was not in response to toxicity of TAC.

The behavioral tests indicated that TAC did not affect sensorimotor coordination, muscle tone, strength, or reflex reaction. Learning and memory can be considered sensitive behavioral tests of toxicity in that slight changes in ability to perceive stimuli can alter rates of acquisition and retention test performance, memory processing, or recall. Because acquisition (i.e., learning) was not adversely affected by TAC and because memory retention was enhanced after 4 and 6 months of ingesting TAC, the drug could not have had a meaningfully toxic effect on sensorimotor systems, learning, memory processing, or recall.

Although results from a mouse model are difficult to extrapolate directly to humans, these data may have a bearing on the clinical potential of TAC to improve memory. Recent preliminary TAC studies in humans (Summers et al., 1981; 1986) suggest that acute parenteral and oral TAC may exert a beneficial effect on learning and memory. The present findings with mice confirm not only the drug’s sustained beneficial effect on cognition but also the drug’s long-term lack of chronic hepatotoxicity or side effects. In addition, the practicality of long-term oral administration of an effective memory-enhancing dosage is demonstrated.

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