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Behavioral and Neural Toxicity of Arteether in Rats

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GENOVESE, R. F., D. B. NEWMAN, J. M. PETRAS AND T. G. BREWER. *Behavioral and neural toxicity of arteether in rats.* PHARMACOL BIOCHEM BEHAV **60**(2) 449–458, 1998.—Repeated administration of the artemisinin antimalarial compound, β-arteether (AE) (25 mg/kg, IM) was evaluated in rats using a two-choice, discrete trial, auditory discrimination task and subsequent neurohistology. Rats were trained to choose one of two response levers following presentation of white noise or a tone + white noise. Increasing and decreasing the intensity of the tone increased and decreased discriminability, respectively, and differential reinforcement density produced systematic changes in response bias. AE (n = 5) or vehicle (n = 5) was injected daily (9–12 days). Initial injections of AE did not affect behavioral performance. Continuing daily injections produced significant decreases in choice accuracy and significant increases in choice reaction time. When overt signs of severe toxicity were observed, rats were sacrificed and significant neural pathology was observed in the nucleus trapezoideus of AE-treated rats. In a subsequent experiment, AE was injected for 3 (n = 5), 5 (n = 5), or 7 (n = 5), consecutive days and performance was examined for an additional 7 days. Behavioral disruption was only observed in rats receiving AE for 7 days and the greatest degree of disruption occurred after AE injections were completed. Histopathological examination showed significant neural pathology in the nuclei trapezoideus, superior olive, and ruber of rats receiving 7- and 5-day AE regimens, and in the nucleus trapezoideus of rats receiving the 3-day regimen. Thus, behavioral disruption reflected, but did not predict, neuropathology. These results confirm and extend earlier results demonstrating neurotoxicity of AE in rats. Further, these results demonstrate that the auditory discrimination task provides an objective behavioral measure of AE neurotoxicity, and thus, can serve as a valuable tool for the safety development of AE and other artemisinin antimalarial compounds. © 1998 Elsevier Science Inc.

Antimalarial Artemisinin Audition Discrimination Neuropathology Safety assessment

ARTEMISININ (Qinghaosu) is the principal medicinal of the Chinese herb *Artemisia annua* L., and is an effective antimalarial agent (15,18). As the instances of multidrug resistance to malaria producing parasites (e.g., *Plasmodium falciparum*) increase, interest in artemisinin compounds has grown [see (19,26)]. To improve the antimalarial efficacy of artemisinin, several derivatives have been synthesized (14,25). Among these derivatives is β-arteether (AE), the ethyl ether analogue of dihydroartemisinin. AE demonstrates substantial antimalarial activity [(e.g., (24)] and is currently undergoing advanced clinical development (7,16).

Initially, the toxicity of artemisinin and its derivatives, including AE, was believed to be relatively low (6,15). More recent evidence, however, shows that high dose AE produces a distinctive and progressive syndrome of neuropathology in rats, dogs and rhesus monkeys (1,2,10,17,22). That is, repeated AE administration can produce substantial CNS neuropathology, with the brainstem selectively affected. The method by which AE produces neural damage is unclear. Some evidence suggests that the mode of action for the antimalarial efficacy, and also the neurotoxicity of artemisinin compounds, is free radical formation (20,21).

Early detection of AE neurotoxicity is problematic because gross behavioral symptoms, such as tremor and gait disturbance, following repeated high-dose AE administration, are only observed shortly before death. Thus, there is a paucity of objective behavioral measures of AE-induced toxicity. The ability to measure behavioral changes that reflect or, ide-

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ally, predict, neuropathology would be a valuable aid for the development and safety assessment of AE and other artemisinin derivatives.

We have previously found that, in rats, repeated AE administration produces dose-dependent neuropathology and that the auditory brainstem structures (3), nucleus trapezoideus (n. trap.) and nucleus superior olive (n. sup. o.) are particularly affected (9,10). In this regard, our results are generally consistent with results obtained by Brewer et al. (1,2). Additionally, we have previously reported that repeated AE administration failed to produce behavioral disruption in rats under a simple schedule of operant conditioning (VI-18 s), although subsequent histopathological analysis revealed substantial neuropathology in the n. trap. (10). Because brainstem auditory system structures have consistently been shown to be sensitive to AE-induced neuropathology, auditory dysfunction may be a reasonable indicator, and possibly a predictor, of AE toxicity. Therefore, the present study assessed the effects of AE on a behavioral task that is dependent upon audition. Specifically, we assessed the effects of repeated AE administration in rats, using a two-choice, discrete trial, discrimination task where the discriminative stimuli were white noise and a tone plus white noise. Subsequently, histological assessments of various brainstem nuclei were made with specific emphasis on the auditory nuclei of the trapezoid body and the superior olive.

METHOD

Subjects

Adult male Sprague–Dawley rats (Charles River, Wilmington, MA) were used. Rats were individually housed under a 12 L:12 D cycle (lights on at 0600 h) and water was always available in the home cages. Food was restricted to that earned during behavioral sessions as well as supplemental feedings (Agway Pro Lab Rodent Chow) to maintain body weights at approximately 320 g.

Apparatus

Behavioral testing was conducted using eight standard rodent operant conditioning chambers (Coulbourn Instruments, Allentown, PA, Model E-10-10 or equivalent) measuring 38 × 46 × 32 cm with Plexiglas walls (other than a stainless steel front wall) and ceiling and stainless steel bars as the floor. Each chamber was outfitted with two response levers, a speaker module, stimulus lights mounted above each response lever, and a food dispenser to deliver 45 mg food pellets (Bio-Serve, Frenchtown, NJ). Chambers were housed in ventilated, sound- and light-attenuating cubicles (Coulbourn Instruments). Supplementary sound attenuation was provided by lining each cubicle with sound dampening foam (Scosche Industries, Moorpark, CA). The speaker in each chamber was attached to a mixer/amplifier (Coulbourn Instruments, Model S82-24) with inputs from sound generators that produced white noise (10–10 kHz) (Coulbourn Instruments, Model S81-02) or precision sine waves (Coulbourn Instruments, Model S81-06). Sound pressure level (SPL) was determined with a sound level meter (Edmund Scientific, Model N38732) and was measured inside the operant chamber at a fixed point approximately 5 cm from the front of the speaker panel. SPL was checked regularly and only minor adjustments were necessary. Experimental events were controlled and monitored using a PDP-11/73 microcomputer (Digital Equipment Corporation, Maynard, MA), using the SKED (State Systems, Kalamazoo, Ml) control language.

Behavioral Procedure

Rats were trained on a two-choice, discrete-trial auditory discrimination task. Behavioral sessions were normally conducted daily (Monday–Friday), or daily during drug administration phases, and were approximately 60 min in duration. Each session consisted of a series of discrete trials during which a discriminative stimulus (either white noise or tone plus white noise) was presented. The frequency of the tone was 750 Hz, and the SPL was normally 75 dB. The SPL of the noise was normally 70 dB. The frequency of the tone and the SPL of the tone and the noise have been shown to be discriminable by rats using traditional threshold procedures (11,12). Figure 1 diagrams the flow of the procedure. Both stimulus lights (located above the response levers) were illuminated at the time of auditory stimulus presentation. Thus, the stimulus lights signaled the opportunity for reinforcement, and the discriminative stimulus (auditory stimulus) signaled which lever would produce reinforcement. Trials (i.e., noise or tone plus noise) were randomly determined with an equal probability of presentation of each discriminative stimulus. The stimulus lights and auditory stimuli were presented for 10 s or until a choice response occurred. If no choice was made within the presentation period, the trial was terminated and scored as a "no decision." The houselight and stimulus lights were extinguished upon termination of the stimulus presentation period. If the choice response was correct (i.e., a left lever press during presentation of the noise alone or a right lever press during presentation of the tone plus noise) then food (normally a single 45 mg pellet) was delivered and a food trough light was illuminated for 1 s. Stimulus presentation periods were separated by an intertrial interval (ITI). If a correct choice response, or no decision, occurred, then the duration of the ITI was scheduled to be 20 s (short ITI). If an incorrect choice occurred, however, the scheduled duration of the ITI was 60 s (long ITI). Responses during the ITI were recorded but had no programmed consequences except that responses during the last 3 s of an ITI increased the length of the ITI by 3 s. Thus, the duration of an ITI could extend beyond its sched-

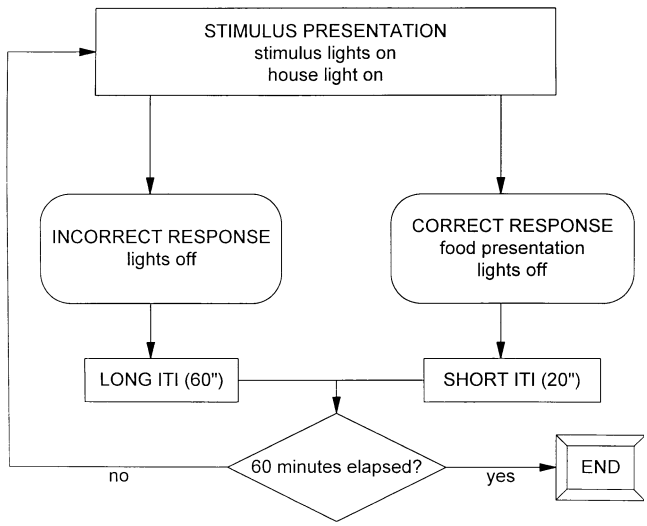

FIG. 1. Flow diagram of the auditory discrimination task (ADT).

uled length. The latter condition was employed to reduce the possibility that a choice response would occur because of proximity to a response lever.

Rats were first trained to lever press for food, during which times the stimulus lights were illuminated and pressing either lever produced a single food pellet. When responding was maintained by food reinforcement, sessions were conducted where the stimulus lights were periodically extinguished, during which time responding did not produce reinforcement. Subsequently, the auditory discrimination procedure was introduced but sessions were conducted where an incorrect choice produced another trial with the same discriminative stimulus (i.e., correction procedure). Finally, the terminal auditory discrimination procedure was used and behavioral and pharmacological manipulations began after stable responding was observed. Stable performance was judged to occur if overall accuracy was above 80% and less than approximately 15% deviation in overall correct responding was observed for at least seven consecutive sessions. In general, all rats reached the criterion of stability after 50–200 sessions. A few rats, however, did not achieve an appropriate level of performance and were not used in this study.

EXPERIMENT 1

To verify that behavioral control was established by the auditory discrimination model, the effects of manipulations in reinforcement density and stimulus intensity were evaluated. We first manipulated the number of food pellets delivered following correct choices during the noise and tone plus noise stimulus trials. Normally, a single pellet was delivered for all correct choices. In the first manipulation, three food pellets were delivered following correct choices on noise trials for three consecutive sessions. Subsequently, the single pellet reward condition was reinstated for five sessions. Next, three pellets were delivered following correct choices on signal plus noise trials for three consecutive sessions. Subsequently, the single pellet reward condition was again reinstated.

Following reinforcement density manipulation, we manipulated the intensity of the signal on signal plus noise trials. The SPL of the 750 Hz tone, which was normally 75 dB, was changed during single sessions to 70, 65, and 85 dB, respectively. At least two sessions where the SPL of the tone was returned to 75 dB separated the tone intensity manipulations. Subsequently, sessions were conducted under normal stimulus intensities and reinforcement densities for several weeks, and then pharmacological manipulations began (in Experiment 2).

Results and Discussion

In general, response accuracy was stable in all rats and typically overall percent correct was above 85%. Typically, 135–150 trials were completed during each session and the number of no decisions during a session was normally less than 5. Most rats, however, were observed to have a response bias in that correct responding during the two types of stimuli were not equal. Response bias, however, was stable within rats, but not systematic between rats. We did not attempt to investigate the nature of the differences because lever position preferences are common in two-choice discrimination paradigms. Choice responses were typically made on nearly every stimulus trial during a session. That is, the number of no-decisions during a session rarely exceeded five for any rat. Reaction time was also observed to be stable and typically the average session reaction time was approximately 1 s. In general, responding during ITI components during the session was relatively infrequent. For a few rats, however, a substantial number of responses during the ITI (both short and long) were consistently observed.

When reinforcement density was changed such that three pellets were delivered following correct responses during noise trials, choice responding changed quickly and dramatically. That is, there was a large increase in the number of trials when a "noise-correct" response occurred. Thus, accuracy during tone plus noise trials decreased, while accuracy during noise trials increased. The latter effect, however, was limited to the extent that baseline accuracy was normally very high. When reinforcement density was changed such that three pellets were delivered following correct responses during noise plus tone trials, an opposite effect was observed. That is, a large increase in the number of trials when a "tone plus noise-correct" response occurred. Under these conditions, accuracy during noise trials decreased, while accuracy during tone plus noise trials increased. Again, the latter effect was limited to the extent that baseline accuracy was normally very high. When reinforcement density was again equal (i.e., a single food pellet for correct choice during both types of trials), premanipulation responding quickly returned. Figure 2 shows the effects of manipulating the number of food pellet rewards in a single, representative, rat.

In general, decreasing the tone SPL from 75 dB to 70 dB had very little effect in most rats, although small decreases in overall accuracy were sometimes observed. When SPL of the tone was decreased to 65 dB, however, a dramatic decrease in choice accuracy was observed in all rats. Furthermore, accuracy during noise plus tone trials was typically decreased to a

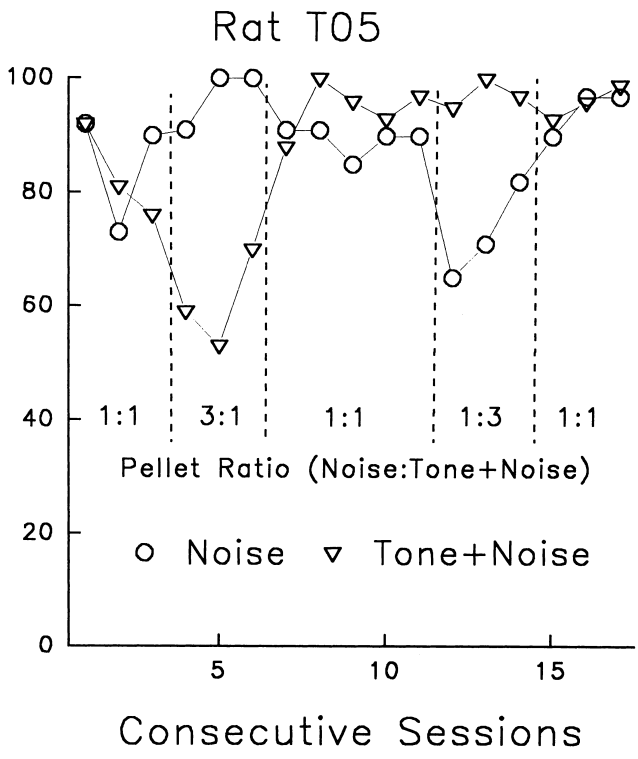

FIG. 2. Effects of varying the amount of reward (number of food pellets delivered) for correct choices to the noise and tone + noise stimuli on accuracy (percent correct) in a single rat. Circles and triangles represent accuracy on noise and tone + noise trials, respectively. Reward ratio is indicated numerically and delineated by diagonal lines.

far greater extent than accuracy during noise trials. Increasing the tone SPL to 85 dB also produced a substantial effect in most rats. That is, overall accuracy increased and, in general, accuracy on tone plus noise trials was increased to a greater extent than during noise trials. When SPL of the tone was returned to the baseline value of 75 dB, premanipulation responding quickly returned. Figure 3 presents the effects of manipulating the SPL of the tone on tone plus noise trials in a single, representative, rat.

These results are consistent with Signal Detection theory (13) in that sensitivity and response bias were under the control of the physical characteristics of the stimulus and the relative consequences of the response, respectively. Specifically, choice accuracy could be increased or decreased by increasing or decreasing, respectively, the SPL of the tone. Further, accuracy on noise and tone plus noise trials could be differentially manipulated by selectively changing the density of reward for correct responses following either one of the two discriminative stimuli. Thus, response bias was affected by altering reinforcement density. These manipulations demonstrate that performance was under the stimulus control of the auditory discrimination procedure [see (8)].

EXPERIMENT 2

Following behavioral manipulations in Experiment 1, we assessed the effects of repeated administration of AE in the same rats. We chose to use a dose of AE, 25 mg/kg, that previous research (1,2) has demonstrated to produce gross behavioral effects if administered daily. A comparable group was administered vehicle during the same time period. Subsequent to AE or vehicle administration, rats were sacrificed, and histology was performed on selected brainstem nuclei.

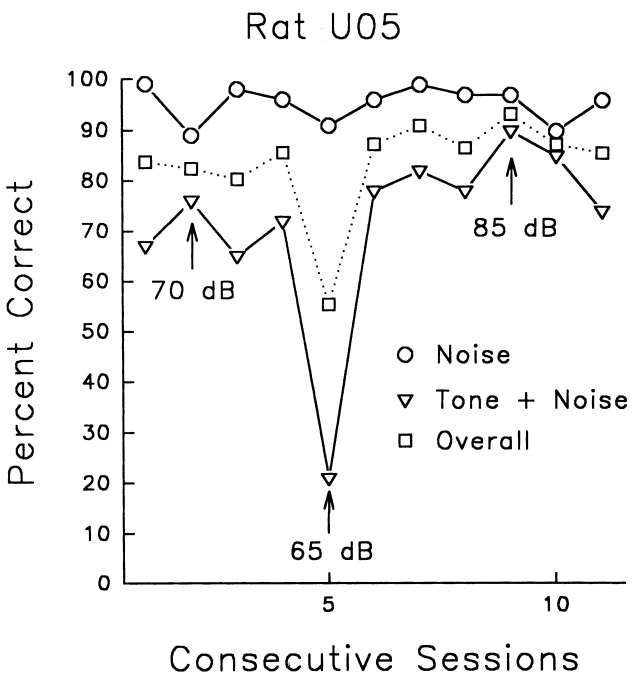

FIG. 3. Effects of varying the SPL of the tone on accuracy (percent correct) in a single rat. Circles and triangles represent accuracy on noise and tone + noise trials, respectively. Squares represent overall accuracy. Arrows indicate sessions where the SPL of the tone was changed from 75 to 70 dB and 65 and 85 dB, respectively.

Pharmacological Procedure

β-arteether was obtained under contract to the U.S. Army, formulated in sterile sesame oil, and sterile sesame oil was used for vehicle injections. Rats were divided into two groups of five, matched on the basis of overall choice accuracy. One group received daily injections of 25 mg/kg AE, while the second group received daily injections of vehicle. Drug and vehicle injections were given IM about the thigh muscle (alternating legs daily) in a volume of 0.5 ml/kg body weight, using a 28-gauge needle. Injections were given approximately 30 min before the start of the session. Each rat was administered drug or vehicle for 9–12 consecutive days. Rats were euthanized in pairs (i.e., one vehicle-treated and one AE-treated) after the session that followed the final injection. The determination of the number of total injections was made by visual assessment of signs of overt toxicity for each AE-treated rat. In this respect, one rat expired overnight following the 11th AE injection, and had to be excluded from subsequent histopathological analysis.

Histological Procedure

All histology was performed without prior knowledge of the treatment conditions. Rats were sacrificed with lethal injections of pentobarbital sodium (100 mg, IP). Thoracotomy exposed the pericardium, which was opened and the right atrium was pierced to permit exsanguination. Transcardiac perfusion was then performed first using a buffered clearing solution [modified from (27)], followed by the fixative solution (Bouin's fluid). The central nervous system remained in situ for 2 h before dissection to avoid the development of neuronal hyperchromatosis [see (4,5)]. Whole brains were then removed and immersed in fresh Bouin's fluid for approximately 6 h. Brains were then blocked transversely and immersed in daily changes of ethanol (70%) to remove excess picric acid. Dehydration in ascending grades of ethanol followed, and clearing was accomplished using cedarwood oil followed by brief treatment with xylene before paraffin embedding. A rotary microtome was used to cut and collect 10 μm serial sections. Three serial sections sets were acquired and stained according to the methods of Nissl (cresylechtviolett; CV), Kluver-Barrera (luxol fast blue, and cresylechtviolett counterstained; KB), and hematoxylin and eosin (H&E).

All section sets (CV, KB, and H&E), through the medulla oblongata and pons, were examined microscopically for evidence of cellular pathology in the n. trap. of the auditory system and the nucleus nervi facialis (n. fac.) as a control, because previous studies have shown that this nuclei is undamaged by AE. Numerical data were obtained from cresylechtviolett, KB, or H&E stained brain sections, separated by no less than 110 μ to 229 μm, which were scanned systematically. Neurons with visible nucleoli were counted until the target of 100 or more cells were identified (sum of affected and unaffected neurons). A percent of affected neurons (e.g., chromatolytic neurons) score was calculated as the ratio of affected neurons (numerator) to total neurons (denominator) × 100. In the absence of neuronal injury the structure under study was scored as unaffected (i.e., given a value of 0 percent affected).

Data Analysis

When a response or an experimental event occurred, the elapsed time within the session was recorded. From these data, the following measures were calculated for each session: overall percent correct (and, thus, number of food pellets pre-

sented), percent correct on noise and tone plus noise trials, average reaction time (i.e., the time from the onset of the auditory stimulus to a response), number of no decisions, and the number of response during ITI periods (short and long).

To assess behavioral changes following injections, repeated measures *t*-tests (two tailed) were performed (SAS statistical software, Cary, NC) testing the statistical significance of the differences between behavioral measures on the last session before injection and the session following the last injection. To assess histopathological differences, Students *t*-tests (two tailed) were performed (SAS statistical software, Cary, NC) comparing the percentage of affected neurons between AE- and vehicle-treated groups.

Results and Discussion

At first, daily injections of AE had very little or no effect on measures of behavioral performance on the auditory discrimination in all rats. After seven or more injections, however, AE produced substantial disruption of performance in all rats. When visual observation revealed severe overt signs of toxicity, including tremor, jerking limb movements, and ataxia, the rat was sacrificed. No substantial decrease in body weight was observed in any of the rats during the experiment. Figure 4 presents the effects of repeated AE administration on behavioral performance in a single, representative, rat. As can be seen in Fig. 4 (top), AE decreased overall accuracy on the auditory discrimination and increased reaction time (bottom). In some rats (e.g., Fig. 4, middle panel), AE had little effect on the number of responses until the last or next to last dose administered. There was, however, a tendency for AE to increase responding during ITI periods and decrease responding during stimulus periods.

Figure 5 shows the average accuracy of discrimination performance (top) and reaction time (bottom), before and after, repeated AE or vehicle administration. AE produced a statistically significant decrease in accuracy ($p < 0.01$) and increase in reaction time ($p < 0.05$). Decreases in accuracy were not consistently stimulus-specific across rats, or between different sessions for individual rats. That is, response accuracy decreases were observed to occur during both noise and signal plus noise trials. The difference in the number of responses during stimulus presentation were also significantly decreased ($p < 0.01$), but the difference in total session responses was not significantly affected (data not shown). In contrast, no significant differences in accuracy, reaction time, number of responses during stimulus presentation, or total session responses, in vehicle-treated rats were observed ($ps > 0.1$).

In contrast to a previous study (1), we did not observe systematic decreases in body weight in rats receiving AE. Additionally, we noted that all rats would readily eat food pellets earned during the experimental session, and also during supplemental feedings occurring after experimental sessions. The latter result indicates that it is unlikely that a specific flavor aversion or general anorectic effect occurred from AE administration. Rats in the present experiment, however, were food restricted, whereas rats used by Brewer et al. (1) were free feeding. The procedural differences between the two studies may account for the difference in results regarding effects on body weight.

As shown in Fig. 6, microscopic examination revealed bilateral injury in the n. trap. in all AE-treated rats. Pathology varied between central chromatolysis, necrosis, and loss of neurons. Chromatolytic neurons were characterized by a swollen soma, loss of Nissl substance, and by an eccentric cell

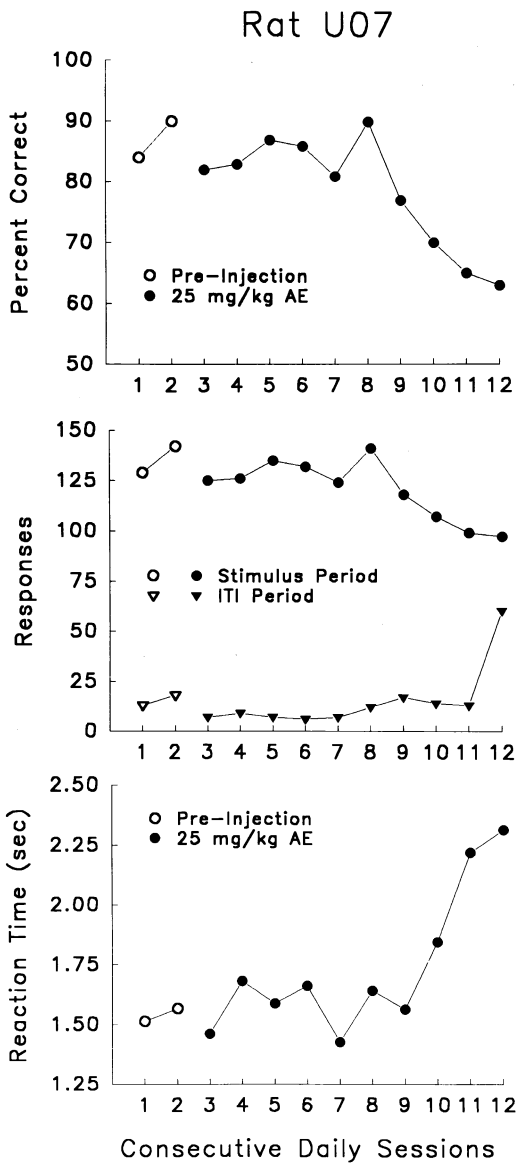

FIG. 4. Effects of repeated daily administration of 25 mg/kg AE on auditory discrimination performance in a single rat. Open symbols represent data from predrug control sessions and closed symbols represent data from sessions, before which, AE was injected. Ordinates: top, accuracy as average percent correct on noise and tone + noise trials; middle, number of responses occurring during stimulus presentation periods (circles) and ITI periods (short + long); bottom, average reaction time to respond to the auditory stimuli.

nucleus. Necrotic neurons exhibited a bright pink-colored cytosol (H&E stain) and karyorrhexis. Loss of neurons was typical and accompanied by diffuse gliosis. Central chromatolysis was severe within the remaining cellular population. In contrast, no pathology was found in the n. fac. of any AE-treated rats.

Microscopic examination of vehicle-treated rats did not reveal any pathology in the n. trap. or the n. fac.. Comparisons of the percent affected neurons between AE- and vehicle-treated rats revealed a statistically significant difference for the n. trap. ($p < 0.01$) but not for the n. fac. ($p > 0.1$) (see Fig. 6).

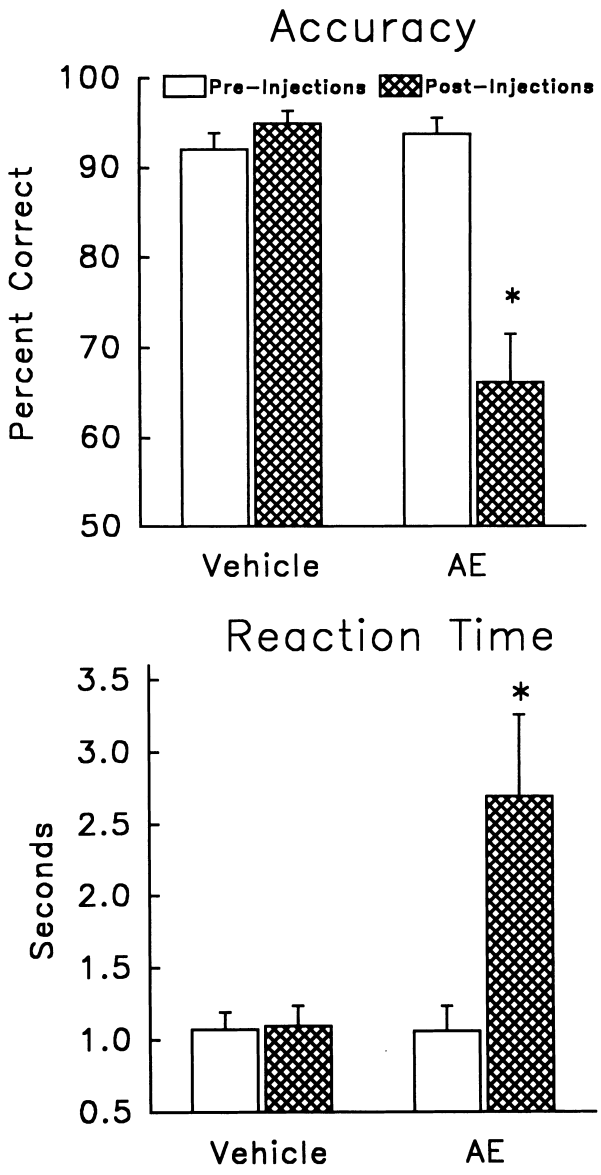

FIG. 5. Average effects of AE (right) or vehicle (left) on accuracy (top) and reaction time (bottom) on the auditory discrimination task in rats. Each bar represents the mean of five rats. Vertical lines about each bar represent the SEM. Open bars represent performance on the day before injection and shaded bars represents performance on the day of the last injection [*-indicates that the difference between pre- and postinjection measures are statistically significant (accuracy, $p < 0.01$; reaction time, $p < 0.05$)].

In rats, AE produced profound performance deficits on an auditory discrimination task. Further, performance degradation correlated with neuropathology in a brainstem auditory structure. In general, our histopathological results confirm and extend earlier findings demonstrating AE-induced brainstem neuropathology (1,2,22). These results also demonstrate a behavioral deficit which has not previously been quantified with artemisinin compounds. In this respect, our results suggest that auditory function, as measured by the ADT, is a valuable behavioral indicator of AE toxicity.

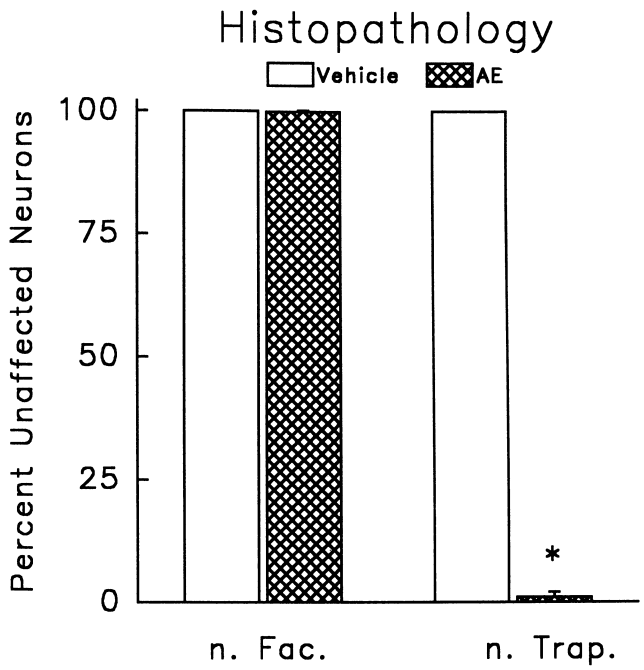

FIG. 6. Average percent of examined neurons judged to be unaffected in the n. facialis. (n. Fac.) and nucleus trapazoideus (n. Trap) in rats treated with AE (shaded bars, $n = 4$) or vehicle (open bars, $n = 5$). Vertical lines about each bar represent the SEM [*-indicates that the difference between AE- and vehicle-treated groups is statistically significant ($p < 0.01$.]

EXPERIMENT 3

In Experiment 3, we investigated the relationship between the number of daily AE (25 mg/kg) administrations and behavioral disruption on the ADT and brainstem histopathology. Because the final days of the AE regimen used in Experiment 2 produced gross behavioral effects, we used smaller regimens in Experiment 3. Specifically, we examined the behavioral and histopathological effects of 3-, 5-, and 7-day, administration of 25 mg/kg AE. We were particularly interested in evaluating the sensitivity of the ADT to reflect or predict AE-induced neuropathology. To increase the possibility of detecting neuropathology, as well as behavioral toxicity, rats were sacrificed for histology 7 days following the last AE administration, in contrast to the procedure in Experiment 2 where rats were sacrificed following the final AE injection. Additionally, histopathological assessment was expanded and included the n. trap., n. sup. o., nucleus ruber (n. rub.), and the n. fac.

Method

Animals. Adult male Sprague–Dawley rats were used as described in Experiment 1.

Behavioral procedure. Rats were trained on the ADT as described in Experiment 1. When stable baseline performance was established, rats were divided into four groups of five rats each. Animals were matched on the basis of overall accuracy on the ADT so that average group accuracy was similar.

Pharmacological procedure. AE or sesame oil vehicle was prepared as described in Experiment 2. Rats were given injections of 25 mg/kg AE for 3 ($n = 5$), 5 ($n = 5$), or 7($n = 5$) consecutive days. A fourth group ($n = 5$) received vehicle injections for 7 consecutive days. Injections were administered 30

min before ADT sessions. Daily ADT sessions were conducted in all rats for 7 days following the final AE or vehicle administrations. Subsequently, rats were sacrificed and brainstem histology was performed.

Histological procedure. All histology was performed without prior knowledge of the treatment conditions. Rats were sacrificed and brains were removed as described for Experiment 1. Brains were then blocked sagittally and immersed in daily changes of ethanol (70%) to remove excess picric acid. A freezing microtome was used to cut and collect 40 μm serial sagittal sections. Sections were stained with a Nissl stain (cresyl violet). One rat died before the behavioral assessments were complete and histological assessment was not possible.

All section sets through the brainstem were examined microscopically for cellular pathology in the n. trap., n. sup. o., n. rub., and the n. fac. (as a control). Numerical data representing the percentage of affected cells for each were obtained as described in Experiment 2.

Data Analysis. Behavioral measures characterizing performance on the ADT were as described in Experiment 1. Specifically, percent correct choice, average choice response time, and the number of stimulus responses were calculated for each day. If less than 10 choice responses were made in a single session, percent correct and reaction time values were not calculated during that session. The latter condition was only observed in rats in the 7-day AE treatment group and only during the final sessions when responding was completely suppressed.

Repeated-measures ANOVA (SAS statistical software, Cary, NC) was used to assess the differences in percent correct, reaction time, and number of stimulus responses during the drug and postdrug sessions for all four treatment groups. Because responding was severely suppressed during the last several sessions in most of the rats in the 7-day AE treatment, only descriptive statistics were used to evaluate measures of percent correct and reaction time for this condition. When a significant F-value was obtained ($p < 0.05$), multiple contrast tests were conducted comparing performance on the first day of treatment with that of the last day of the experiment. For the latter tests, statistical significance was designated if $p < 0.05$.

ANOVA was used to assess the differences in histopathological cell scores for each of the four nuclei examined using the General Linear Models procedure of the SAS statistical software package (SAS statistical software, Cary, NC). When a significant *F*-value was obtained ($p < 0.05$), multiple comparison tests were conducted using Dunnett's *t*, to compare the percentages of affected cells in each of the four nuclei, between drug and vehicle treated groups. For the latter tests, statistical significance was designated if *p*s < 0.05.

Results and Discussion

As observed in Experiments 1 and 2, performance on the ADT was characterized by relatively accurate and stable responding in all rats. As Fig. 7 shows, repeated injections of AE (25 mg/kg) for 3 and 5 days had very little effect on behavioral performance on the ADT. A small trend was observed in that accuracy (i.e., percent correct) tended to be lower in the 5-day AE treatment than the 3-day AE treatment, which was lower than the 7-day vehicle treatment. Differences in average accuracy were greatest during the 7 days following AE and vehicle administration. No statistically significant differences, however, were observed for any of the behavioral measures in these three groups (*p*s > 0.05). Additionally, no weight loss or clearly observable signs of toxicity were seen in any of the rats in the 7-day vehicle, 5-day AE, or 3-day AE treatment conditions.

Figure 8 shows the behavioral performance on the ADT for individual rats in the 7-day AE treatment condition. As observed in Experiment 2, the initial injections of AE produced no observable effects on performance. With continued injections, and during postinjection sessions, however, performance was clearly disrupted in all rats. Specifically, accuracy decreased and reaction time increased. For example, in some rats (e.g., Y07, Z04) percent correct dropped to 65% and below. Reaction time was also substantially changed and was observed to more than double on many sessions for several

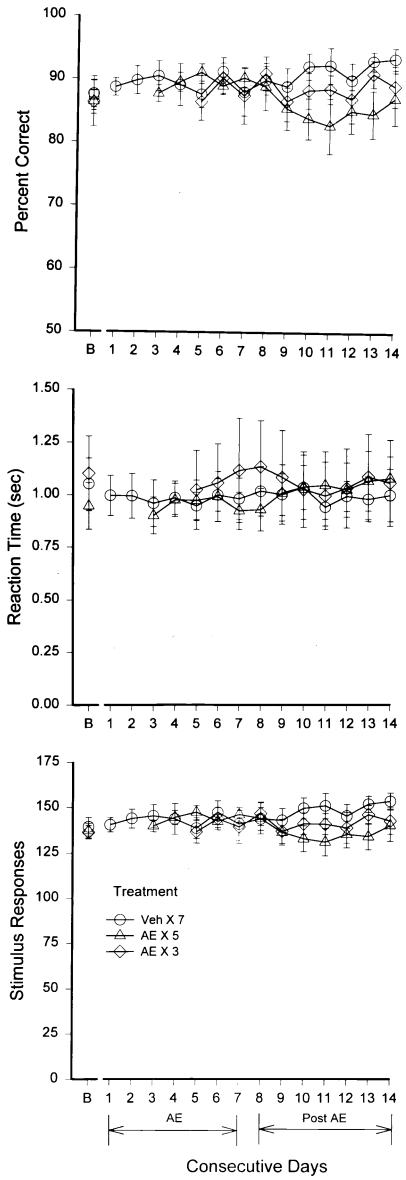

FIG. 7. Average effects of repeated injections of AE (25 mg/kg/day × 3 and 25 mg/kg/day × 5) or vehicle (7 days) on accuracy (top), reaction time (middle), and stimulus responses (bottom) on the ADT in rats. Each point represents the mean of five rats. Vertical lines about each bar represent the SEM. Points above B represent average performance measures for the 10 sessions preceeding injections.

rats. Furthermore, a statistically significant decrease in the number of stimulus responses was observed, $F(14, 56) = 7.89$, $p < 0.001$, and in the case of three rats in this condition, responding was completely suppressed on the eleventh session and beyond (postinjection day 4). Of the latter rats, one expired on the thirteenth session (postinjection day 6). Considerable variability between rats in the time and degree of performance disruption was observed. In general, however, the greatest degree of disruption was observed during sessions following the last AE administration. In this respect, multiple contrasts indicated a significant difference in the number of stimulus responses between the first session and sessions 11–14, Fs$(1, 4) \geq 12.87$, ps < 0.05. Therefore, these results indicate that AE behavioral toxicity is both cumulative and progressive. When responding was severely or completely suppressed, rats were also observed to exhibit tremor and gross gait impairments.

In general, the pattern of performance impairment observed was similar to that observed in Experiment 2. It is notable however, that in the present experiment, greater response suppression was observed than in Experiment 2, even though more AE was administered in Experiment 2. An important difference between the two procedures however, was the 7-day postinjection testing, which was not used in Experiment 2. It is likely, therefore, that performance deficits would have continued to increase in rats used in Experiment 2.

Figure 9 shows the results of histopathological examination of four brainstem nuclei. Substantial neuropathology was not observed in any of the nuclei examined in any of the vehicle-treated rats. Similarly, substantial neuropathology was not observed in the n. fac. in any rats. Statistically significant neuropathology was observed, however, in the n. trap., $F(3, 15) = 15.63$, $p < .001$, n. sup. o., $F(3, 15) = 16.25$, $p < 0.001$, and n. rub., $F(3, 15) = 11.78$, $p < 0.001$. Multiple contrasts (Dunnett's t, ps < 0.05 revealed that all three nuclei (i.e., n. trap.,

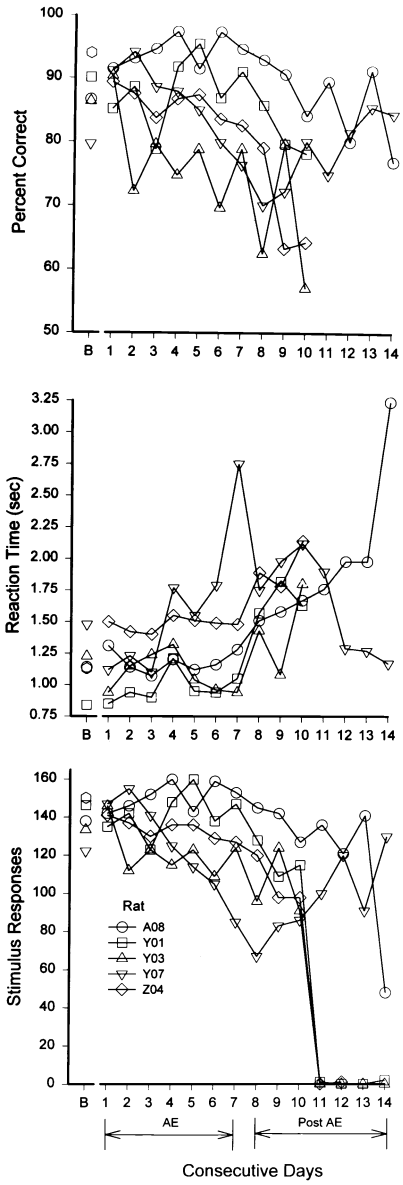

FIG. 8. Effects of repeated injections of AE (25 mg/kg/day × 7) on accuracy (top), reaction time (middle), and stimulus responses (bottom) on the ADT in rats. Each point represents performance from a single rat. Points above B represent average performance measures for the 10 sessions preceeding injections.

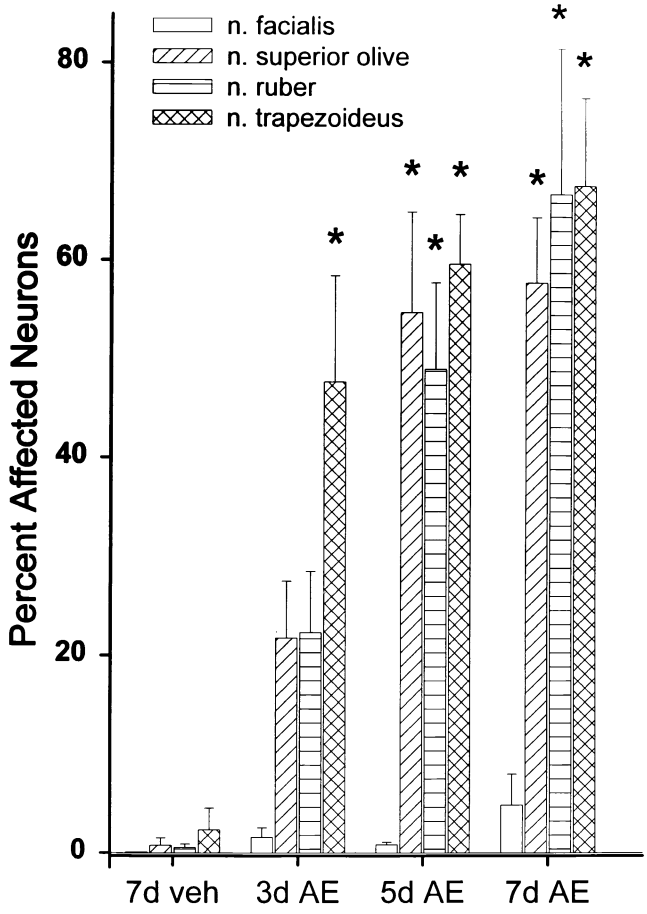

FIG. 9. Average percentage of examined neurons in selected brain stem nuclei judged to be affected following daily administration of AE (25 mg/kg) for 3, 5, or 7 days and vehicle for 7 days. Each bar represents cell counts from the means of five rats ($n = 4$ for 7d AE). Vertical lines about each bar represent the SEM [*-indicates that the difference between nuclei in AE- and vehicle-treated groups is statistically significant ($p < 0.05$)].

n. sup. o. and n. rub.) in 5-day and 7-day AE-treated rats showed significant neuropathology as compared to 7-day vehicle control rats. For the 3-day AE condition, only the n. trap. showed significantly greater neuropathology than 7-day vehicle control condition.

Neuropathology observed in Experiment 3 is consistent with, and extends, the results of Experiment 2. Moreover, the pattern of AE-induced brainstem histopathology in the present experiment was, in general, consistent with earlier studies (1,2,9,10). The present study, however, found that even three consecutive administrations of AE can produce significant neuropathology in the n. trap., and thus suggests that AE neurotoxicity can occur at relatively short duration regimens. These results suggest that, in rats, the n. trap. is particularly sensitive to AE neurotoxicity and, therefore, damage in this area may be the earliest indicator of neuropathology.

In contrast to the histopathologic results of the present study, and previous studies (1,2), Kamchonwongpaison et al. (17), while finding that 50 mg/kg AE for 5 or 6 days was lethal in rats, failed to find any evidence of neuropathology in rats administered 25 mg/kg AE for 5 days or even 30 mg/kg AE for 8 days. It is notable that the latter study used much younger rats, a qualitative histopathologic assay rather than the quantitative method used in the present studies, and sacrificed rats 30 or 50 days following AE injection. It is, therefore, possible that the procedural differences between studies could have accounted for some of the differences in the results. Additionally, the fate of the affected neurons observed in the present study was not systematically studied, and it is conceivable that some recovery could have occurred if more time had elapsed following AE administration. Nevertheless, the disparity in the dose regimens of AE that result in neurotoxicity is surprising and suggests that further research in this area is needed.

Although performance on the ADT was clearly disrupted by AE, it is most notable that performance was only disrupted in rats under the 7-day AE regimen. As in Experiment 2, performance disruption on the ADT served as an objective measure of behavioral toxicity from AE. Neuropathology in brainstem auditory nuclei, however, was observed in rats in both 5-day and 3-day treatment regimens. Therefore, our results indicate that while the ADT provides a measure of behavioral toxicity, it reflects, rather than predicts, neuropathology. That is, it appears that a substantial degree of auditory brainstem neuropathology must occur before behavioral perfomance on the ADT is affected. Clearly, a more sensitive behavioral indicator would be advantageous.

GENERAL DISCUSSION

Under several regimens of administration, AE produced distinctive brainstem neuropathology that included brainstem auditory nuclei. In this respect, our results confirm and extend the finding of previous studies (1,2,9,10,17,23). It is notable that our results indicate that the n. trap. is the most vulnerable of the nuclei assessed and, further, that a shorter daily regimen can produce neuropathology than previously demonstrated.

In the present study, an auditory discrimination task was developed to assess the behavioral toxicity of arteether. The ADT controls performance on the basis of auditory stimuli and response reinforcement, and provides objective measures of performance. In contrast to an earlier study in our laboratories using operant conditioning (10), repeated administration of AE produces clear and systematic disruption of performance (i.e., behavioral toxicity) on the ADT in that accuracy is decreased and reaction time is increased. Furthermore, AE-induced behavioral toxicity is delayed and progressive and in more severe instances produces a complete cessation of responding. The ADT, therefore, offers advantages over subjective ratings of neurological deficits, and can be a useful tool for further study of arteether and other artemisinin antimalarial compounds. In its current form, however, the ADT does not predict, but rather reflects, the onset of brainstem neuropathology because neuropathology in brainstem auditory nuclei was observed in the absence of behavioral disruption.

The detection and prediction of arteether toxicity remains problematic and hampers the development of arteether and other artemisinin drugs for the treatment of malaria. In this respect, studies are currently underway in our laboratories to evaluate behavioral performance that may be more closely dependent upon the functions of those brainstem nuclei that are most vulnerable to the effects of AE and other artemisinin compounds.

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ln conducting the research described in this report, the investigators adhere to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4-3, AR 360-5). The authors thank Sgt. L. Simmons, B. Fantegrossi, K. Gordon, D. Shapiro, M. Vane, Dr. J. O. Peggins, and M. Sperry, for expert technical assistance with the conduct of this research. This work was supported, in part, by WHO Tropical Disease Research Grants 950304, 870388, and 920548.

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