Comparative Resistance of *Anopheles albimanus* and *Aedes aegypti* to N,N-Diethyl-3-methylbenzamide (Deet) and 2-Methylpiperidinyl-3-cyclohexen-1-carboxamide (AI3-37220) in Laboratory Human- Volunteer Repellent Assays

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**ABSTRACT** The insect repellents N,N-diethyl-3-methylbenzamide (Deet) and the racemate and 1S,2'S stereoisomer of 2-methylpiperidinyl-3-cyclohexene-1-carboxamide (AI3-37220) were tested against *Anopheles albimanus* Wiedemann and *Aedes aegypti* (L.) in laboratory human-volunteer assays. Estimated skin doses of Deet or racemic AI3–37220 required to reduce biting by 95% in *Ae. aegypti* were 2.3 and 3.5 × 10⁻² μmol/cm² skin, respectively, whereas estimated doses for 95% bite reduction of *An. albimanus* in an ≈40-yr-old laboratory colony established from El Salvador were 5 times higher at 12 × 10⁻² μmol Deet/cm² skin and >20 × 10⁻² μmol/cm² skin for AI3-37220. In tests with the 1S,2'S stereoisomer of AI3-37220, a newly established colony of *An. albimanus* from Belize bit less aggressively than El Salvador *An. albimanus*. However, the Belize-derived mosquitoes were as resistant as the old El Salvador colony to repellent effects of 1S,2'S stereoisomer of 2-methylpiperidinyl-3-cyclohexene-1-carboxamide. Earlier workers surmised that usual skin doses of Deet would offer only limited protection against *An. albimanus* in the field. Our findings support this speculation, but they also indicate that doses of Deet higher than those needed for protection against *Ae. aegypti* might offer reasonable protection against *An. albimanus*. Results indicate that neither racemate nor 1S,2'S stereoisomer of 2-methylpiperidinyl-3-cyclohexene-1-carboxamide offer as much protection as Deet against *An. albimanus*, despite being highly effective against *Ae. aegypti*.

**KEY WORDS** N,N-diethyl-3-methylbenzamide, insect repellent, (1S,2'S)-2-methylpiperidinyl-3-cyclohexen-1-carboxamide

*Anopheles albimanus* Wiedemann is a vector of malaria that has a wide distribution that includes lowland Middle America and the Caribbean (Belkin et al. 1970, Faran 1980). Results from field and laboratory studies of the sensitivity of *An. albimanus* to Deet have been contradictory. Deet offered high levels of protection against this species in field tests in the Panama Canal zone (Altman 1969), and Deet was more effective than all other candidate repellents in the study. However, laboratory studies by Rutledge et al. (1978) showed that among 18 species and strains tested, *An. albimanus* was the least sensitive to Deet and that this compound offered little protection against biting by this species. Schreck (1985) compiled data from 110 laboratory tests with *An. albimanus* over 10 years at the United States Department of Agriculture laboratory in Gainesville, FL. Although no statistical analysis was performed “because of wide variation in sample sizes,” Shreck (1985) concluded that only limited protection against *An. albimanus* could be expected in the field if using Deet. Ostensibly, the Rutledge et al. (1978) and Shreck (1985) studies were both conducted using the same colony of *An. albimanus* from Gainesville that was established and reared continuously from insects collected in El Salvador in the early 1960s. Thus, tests showing Deet insensitivity in these two laboratory studies may have been biased because they likely used the same El Salvador inbred-laboratory stock, perhaps with a genetic predisposition for resistance to Deet. Given the potential insensitivity of *An. albimanus* to Deet, we designed an experiment using the El Salvador *An. albimanus*, comparing its sensitivity to Deet and to the comparatively newer repellent compound.
Materials and Methods

Mosquitoes. *Ae. aegypti* (red eye Liverpool strain) and *An. albimanus* (El Salvador strain) used in the study were from colonies maintained at the Walter Reed Army Institute of Research (WRAIR). Both colonies were maintained at WRAIR for many years, and both were probably established originally at the United States Department of Agriculture Laboratory in Gainesville (Rutledge et al. 1978). A fresh colony of *An. albimanus* was established from females that were field collected 19–30 August 2002 at multiple sites in Orange Walk District, Belize. The females were given blood meals and transported to the insectary at the Uniformed Services University of the Health Sciences in Bethesda, MD. These females were used to establish a colony, which was reared for five generations. Female progeny from the original wild-caught Belize females were used in the first set of repellent tests (study 1). Because this population was derived from females not fertilized in captivity, they were considered parental stock. Females from the F4 generation were used in the second set of repellent tests (study 2). Insects were reared (Gerber et al. 1994) by feeding larvae ground Tetramin Tropical Fish-food Flakes (Tetra Sales, Blacksburg, VA). Adults were maintained in a photoperiod of 12:12 (L:D) h at 27°C and 80% RH with a cotton pad moistened with 10% aqueous sucrose solution; they were not blood fed.

Chemicals. The repellent compounds 2-methylpiperidinyl-3-cyclohexene-1-carboxamide (AI3-37220) and N,N-diethyl-3-methylbenzamide (Deet) used in the tests were at least 98% pure chemically according to capillary gas-liquid chromatography. Deet and racemic AI3–37220 were obtained from Morflex, Inc. (Greensboro, NC), and the 1S,2’S stereoisomer of AI3-37220 obtained from the Chemicals Affecting Insect Behavior Laboratory where it had been synthesized previously (Klun et al. 2003). For brevity, we refer to racemic AI3-37220 and its 1S,2’S stereoisomer as 220 and SS220, respectively. Deet is a widely used arthropod repellent that is registered with the U.S. Environmental Protection Agency. The compounds 220 and SS220 have been proven to be toxicologically safe for use by humans (Snodgrass 1995, 2000; Snodgrass and Houpt 2002).

Bioassay. In conducting this research, we adhered to the guidelines established by the National Institutes of Health for tests involving human subjects, and protocols were approved by the Human-Use Review Board of the Walter Reed Army Institute of Research.

Study 1 measured the blood feeding (biting) frequency of *Ae. aegypti* and El Salvador *An. albimanus* mosquitoes in response to 0.0 (control), 2.4, 4.8, 9.6, and 19.2 × 10⁻² μmol/cm² skin doses of Deet (treatments 1–5) and 220 (treatments 6–10) applied to human volunteers. Treatments 1 and 6 served as controls (skin treated with ethanol alone). Bioassays were conducted using K & D modules and methods described by Klun and Debboun (2000). A volunteer wearing short pants was seated. Using a skin-marking template and a washable-ink marker, skin areas representing five 3 by 4-cm floor openings of the K & D module were outlined on the outer, top, and inner positions of each thigh. Positions on the thigh treated with Deet or 220 were randomly selected. Locations for treatments 1–5 of Deet or treatments 6–10 of 220 within positions were randomized and labeled numerically. All treatments were pipetted onto a 4 by 5-cm rectangular area 0.5 cm outside of the template marks of the volunteers’ skin in 55 μl of ethanol/treatment. Treating outside template marks assured that areas beneath each K & D module cell contained no untreated skin. Each of five adjacent cells in the K & D modules were provided with five female mosquitoes randomly selected from cages containing ≈200 adults. Mated nulliparous females (5–15 d old) had access only to water 24 h before testing. The K & D module was positioned with the cells aligned over the marked and treated areas of skin. Sliding doors between the cell and skin were then opened. For the next 2 min, the number of females biting (proboscis inserted into skin and/or observed blood-engorged females) within each of the cells was recorded. The trial was concluded at the end of the 2-min period by closing the sliding doors. Individual mosquitoes were scored as having either fed or not fed during a trial. The bioassays were done in a walk-in incubator (27°C and 80% RH) in ambient fluorescent light from 07:30 hours to 10:30 hours. Observations using *An. albimanus* were replicated 24 times with four volunteers (1,200 total mosquitoes used) and observations with *Ae. aegypti* were replicated 18 times with five volunteers (900 total mosquitoes used). Three of the six volunteers were involved in tests against both species. Mosquitoes were used for only one test and modules were washed with water and detergent after each trial.

Study 2 measured the responses of the newly established *An. albimanus* Belize colony, the El Salvador *An. albimanus* colony, and the red eye Liverpool *Ae. aegypti* colony to either an ethanol control or 2.4 × 10⁻² μmol of SS220/cm² skin, using the same methods described above. SS220 stereoisomer was used in this test because the compound was previously demonstrated (Klun et al. 2001) to be significantly more effective against *Ae. aegypti* than 220 (racemate), and it was surmised that SS220 might likewise be more effective than the racemate against *An. albimanus*. The
SS220 dose was selected because a previous study (Klun et al. 2003) showed that 2.4 × 10⁻² μmol of SS220/cm² reduced An. aegypti biting by at least 50%. Pairs of module cells containing five mosquitoes of each of the three mosquito types were positioned randomly over SS220 treated skin or an area of control skin. Study 2 involved five volunteers (one was in study 1 and four were new volunteers) and 29 replicates (870 total mosquitoes used).

Statistical Methods. In study 1, we modeled the logit of the proportion of mosquitoes not biting as (log (p/(1 − p))), where p is the proportion of mosquitoes not biting) to develop a dose–response curve. This standard transformation has desirable statistical properties for developing dose–response relationships by using data that are samples from a binomial distribution. We found that a straight line relationship between dose and logit (p) was obtained by taking the square root of dose. The log transformation on dose, recommended by Anonymous (1983) did not provide this straight line relationship, although it may be an effective transformation of dose for other mosquito species. Because there can be significant person-to-person differences in the number of mosquitoes biting for the same dose (Gilbert et al. 1966), we included person as a random variable (modeled as drawn from a normally distributed population) in the generalized linear mixed model we developed. Although it is conceivable that other block effects (K & D module replicate, position on thigh, day of trial) existed, a preliminary analysis of this data set (and experience with similar data from other studies) found these effects estimated as zero or near zero; thus, they were not included when modeling these data.

We used PROC NLmixed (SAS Institute 1999) to estimate the parameters of the model (here written for a specific species and compound, so notation for these is suppressed), log \((\pi_i/(1 − \pi_i)) = \beta_0 + \beta_i D_i + V_j\), where \(\pi_i\) is the true proportion of nonbiting mosquitoes for the ith dose level (\(D_i\)) and the jth volunteer (\(V_j\)). The data for both species and both compounds were modeled jointly, by using additional parameters, so that t-tests on these parameters could be made, e.g., to test whether the species’ dose–response curves differed in intercept or slope.

The estimated model parameters were used to create a dose–response model for the two species for each of the compounds and to estimate 95% fiducial limits (Draper and Smith 1981) for the dose required to obtain 95% efficacy (defined as 95% of the mosquitoes not biting) against An. aegypti (Deet and racemate 220) and An. albimanus (Deet only). A fiducial interval is the confidence interval on what is ordinarily the independent variable when making predictions by using what is ordinarily the dependent variable, by using inverse regression. In this experiment, dose (independent variable) was first used to estimate the proportion of mosquitoes not biting (dependent variable). Results from this regression were then used to estimate, for 95% of mosquitoes not biting, the smallest interval (the fiducial interval) that would contain the corresponding true dose 95% of the time.

The data from study 2, by using SS220 against An. aegypti, and Belizian and El Salvadorian colonies of An. albimanus, were treated in a manner similar to those from study 1. Because only a single dose of 2.4 × 10⁻² μmol of SS220/cm² skin was used, the statistical model (for a specific species and compound) reduces to \((\pi_i/(1 − \pi_i)) = \beta_0 + \tau_i + V_j\), with \(\tau_i\) representing the effect of the repellent (versus control, \(i = 0, 1\)) and other symbols as defined above. Thus, any differences between populations and compounds would emerge as differences in intercepts (\(\beta_0\)) and values of \(\tau_i\), tested for significance using a t-test. Point estimates of the \(\beta_0 + \tau_i\), their standard errors, and t-values were calculated using Proc NLmixed.

Results and Discussion

In study 1, An. aegypti and An. albimanus did not differ significantly in their propensity to bite on untreated control skin (t-test, \(df = 20, P = 0.13\)). There were significant effects (t-test, \(df = 20, P < 0.01\)) of both compound and species on the slope of the response to dose, with Deet significantly more effective than 220, and An. aegypti significantly more sensitive to both repellents than An. albimanus. There was no significant interaction (t-test, \(df = 20, P = 0.56\)) between species and dose, i.e., both species responded similarly to the compounds, other than differences in their overall sensitivity to repellent.

A dose–response curve, backtransformed to the original scale (proportion repelled) is shown in Fig. 1. Point estimates for 95% efficacy are 2.3 × 10⁻² to 2.3 × 10⁻² μmol/cm² skin (An. aegypti, Deet), 3.5 × 10⁻² to 4.6 × 10⁻² μmol/cm² skin (An. aegypti, 220), and 12.0 × 10⁻² to 30.0 × 10⁻² μmol/cm² skin (An. albimanus, Deet). Point estimates (and fiducial intervals) for 95% efficacy were not estimated for An. albimanus against 220 because they exceed the highest dose used, i.e., lie outside the range of the data. The 95% fiducial interval on dose for 95% efficacy for An. aegypti was 1.4–3.1 × 10⁻² μmol/cm² skin for Deet and 2.3–4.6 × 10⁻² μmol/cm² skin for 220. This interval for An. albimanus for Deet is 8.7–19.3 × 10⁻² μmol/cm² skin. Using the inverse regression equations, we estimated that a dose of Deet that repels 95% of An. aegypti mosquitoes will only be ≈66% effective against An. albimanus.

Because only six volunteers were used in study 1, the variance attributable to person-to-person differences (variance = 0.15, SE of variance estimate = 0.12, on the transformed scale) was poorly estimated. However, we felt it was important to retain this term in the model because other studies (Gilbert et al. 1966) found large and systematic differences in attractiveness to mosquitoes among the human volunteers used in their study. An additional reason for retaining the term concerns the validity of t-tests if person-to-person differences are ignored. The logit transformation of a proportion produces an approximately normally distributed variable with variance \((m\pi(1 − \pi))^{-1}\) (Cox and Snell 1989). Taking \(\pi \approx 0.95\) (true proportion of mosquitoes not biting) and \(m\) as 20 (degrees of freedom for t-tests above), the variance attributed to
causes other than person-to-person differences at the 95% efficacy is estimated at 1.05. If the variance due to person-to-person differences were ignored, the true variance of the residuals would be larger (0.15 / 1.20) than that assumed based on an uncontaminated binomial distribution. This would make tests of interest (species and compound differences) too liberal, (declaring differences as significant when, in fact, they are not, more than the nominal 5% of the time), similar in effect to ignoring over dispersion in $\chi^2$ tests (Kramer and Schmidhammer 1992).

The results from study 2 are given in Table 1. The propensity to bite in the two An. albimanus colonies differed significantly (t-test, df = 24, $P < 0.01$), but biting was not significantly (t-test, df = 24, $P = 0.51$) affected by the presence of repellent at a dose of $2.4 \times 10^{-2} \mu$mol of SS220/cm$^2$ skin. This is consistent with results from study 1, where, for this dose of 220, percentage of biting only decreased by an estimated 22%.

In contrast, for this dose, biting by Ae. aegypti mosquitoes was suppressed by 90%.

The substantial difference in biting tendency between the two An. albimanus colonies demonstrates that large local intraspecific variation among mosquito populations can exist. This potential variation is typically ignored when testing repellents, and is, in general, poorly understood. We have also often seen large variation in the level of biting propensity among several colonized species in other laboratory repellent studies (unpublished data) that occur from time to time with no explainable reason. Thus, a formulation that repels 95% of mosquitoes in one population at one time may be more or less effective against that same species at a different locality or time. What seems to be consistent is the relative resistance of the two An. albimanus populations to repellents. Thus, relative resistance to the repellents is a species rather than population characteristic. In an earlier study of Deet and SS220 against An. stephensi (Klu n et al. 2003), there was no detectable resistance to either compound, and this is evidence that resistance seen in An. albimanus is not a uniform characteristic in the genus.

Although there was some difference among compounds in effectiveness, with Deet slightly more effective than racemic 220 for both mosquito species, this difference was largely overshadowed by the large species response differences to the compounds. Because protection is desired against all mosquito species a person is exposed to, an effective mosquito repellent should repel the least sensitive species most of the time. Thus, to obtain a meaningful test of repellent effectiveness, the estimated proportion of mosquitoes not biting should be compared with the expected proportion if no repellent were used.

### Table 1. Estimated proportions of Ae. aegypti, An. albimanus (Belize), and An. albimanus (El Salvador) mosquitoes not biting on treated skin ($2.4 \times 10^{-2} \mu$mol SS220/cm$^2$ skin) and untreated skin and estimated bounds on an upper and lower 95% confidence interval

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Species</th>
<th>Origin</th>
<th>Lower 95% Estimate</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>albimanus El Salvador</td>
<td>0.09</td>
<td>0.15</td>
<td>0.24</td>
</tr>
<tr>
<td>SS220</td>
<td>albimanus El Salvador</td>
<td>0.15</td>
<td>0.23</td>
<td>0.34</td>
</tr>
<tr>
<td>Control</td>
<td>albimanus Belize</td>
<td>0.58</td>
<td>0.69</td>
<td>0.79</td>
</tr>
<tr>
<td>SS220</td>
<td>albimanus Belize</td>
<td>0.51</td>
<td>0.63</td>
<td>0.73</td>
</tr>
<tr>
<td>Control</td>
<td>aegypti Liverpool</td>
<td>0.28</td>
<td>0.39</td>
<td>0.51</td>
</tr>
<tr>
<td>SS220</td>
<td>aegypti Liverpool</td>
<td>0.86</td>
<td>0.93</td>
<td>0.96</td>
</tr>
</tbody>
</table>
compounds, trials should be conducted ideally with a variety of mosquito species and from a variety of different locations or origins, with an emphasis on those, like *An. albimanus*, that have demonstrated relative insensitivity to repellent compounds. It will be of interest to learn whether field tests of *An. albimanus* populations at various locations across Middle America reveal the same indications of repellent resistance as seen in our laboratory investigations with the mosquitoes colonized from El Salvador and Belize. In practice, formulated repellents are often applied at dosages far in excess of that used in our laboratory assays. We surmise the failure of formulated products against resistant species such as *An. albimanus* will usually be observed as dramatically reduced protection time, rather than as the complete absence of protection.

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