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A NEW IN VITRO BIOASSAY SYSTEM FOR DISCOVERY OF NOVEL HUMAN-USE MOSQUITO REPELLENTS[1]

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ABSTRACT. A Klun & Debboun (K&D) test module, previously developed and used for quantitative measurement of the efficacy of mosquito repellents on human volunteers, was adapted for in vitro evaluation of repellents by coupling the module with a membrane-blood reservoir. Performance of Deet, Bayrepel®, and SS-220 insect repellents in the new in vitro system was compared with their performance on humans against mosquitoes using our standard in vivo system. For each compound, in vitro dose–response assays were conducted with compounds applied to cloth positioned over blood reservoirs covered with Baudruche membrane against *Aedes aegypti*. The repellents were also tested in vitro against *Anopheles stephensi* and *Ae. aegypti* at a fixed dose of 24 nmol compound/cm^2 cloth over the Baudruche and Edicol collagen membranes. Concurrently, the repellents were tested at the fixed dose using the K&D module on human volunteers. The observed proportions of both mosquito species deterred from biting in the fixed doses in the in vitro assays were similar to those obtained using humans, being clearly able to distinguish controls from repellents, and differing only in the ranking of the effectiveness of some of the repellents. Dose–response relationships of the in vitro and in vivo systems were also very similar, although not directly comparable because the data were not collected concurrently. This new in vitro assay system can be used in high throughput screening of compounds to identify new repellents having potential for use as topical mosquito repellents on humans.

KEY WORDS *Aedes aegypti, Anopheles stephensi,* N,N-diethyl-3-methylbenzamide, (1S, 2′S)-methylpiperidinyl-3-cyclohexen-1-carboxamide, 2-(2-hydroxyethyl)-1-piperidine carboxylic acid 1-methylpropyl ester, yellow fever mosquito, Deet, Bayrepel®, SS-220, mosquito biting

INTRODUCTION

Traditionally, early searches for new topical insect repellents for protection of humans against biting of disease vectors relied on the screening of candidate compounds applied to the skin of human volunteers (Bar-Zeev and Smith 1959). As late as 1970, Schreck et al. (1970) described repellent tests in which compounds of unknown toxicity were applied to the hands of human subjects. Today, this is an unthinkable practice from a human-use safety viewpoint. Although human-biting testing is probably the most effective method to study and characterize repellent compounds (Schreck and McGovern 1989, Collins et al. 1993, Barnard et al. 1998, Klun et al. 2003), it is limited to study of compounds known to be safe for application to humans. This toxicological limitation severely restricts chemical screening programs for discovery of new and effective arthropod repellents for human use. In efforts to overcome this limitation, researchers turned to the use of a variety of test animals, including camels, guinea pigs, rabbits, gerbils, and suckling mice (Schreck 1977, Wirtz et al. 1980) for screening compounds with unknown toxicity. Using laboratory animals in lieu of humans introduces vagary into the screening process because extrapolating experimental results obtained with laboratory animals to humans can lead to erroneous conclusions about repellent efficacy (Rutledge et al. 1994, 1996). In addition, rearing and maintenance of laboratory animals for repellent screening can be regulatorily complex, expensive, and labor intensive. Consequently, efforts were made to develop in vitro screening methods using blood-membrane systems (Bar-Zeev and Smith 1959, Rutledge et al. 1976, Cockcroft et al. 1998); in one case, Sharpington et al. (2000) advocated the use of a wind tunnel system for repellent screening. None of these systems are amenable to high throughput repellent screening. The Rutledge et al. (1976) in vitro blood-feeding system method of repellent testing was recently reevaluated by Rutledge and Gupta (2004). They concluded that the system is in need of significant modification to increase the accuracy, precision, and reliability, and noted that results obtained with it did not always agree closely with repellent-test results observed using human subjects. We report a new in vitro system that yields repellent test results that closely agree with results obtained with humans and can be used to screen large numbers of chemicals and identify novel repellent compounds for human use.

MATERIALS AND METHODS

Insects

Aedes aegypti (L.) and *Anopheles stephensi* Liston used in the bioassays were from colonies main-

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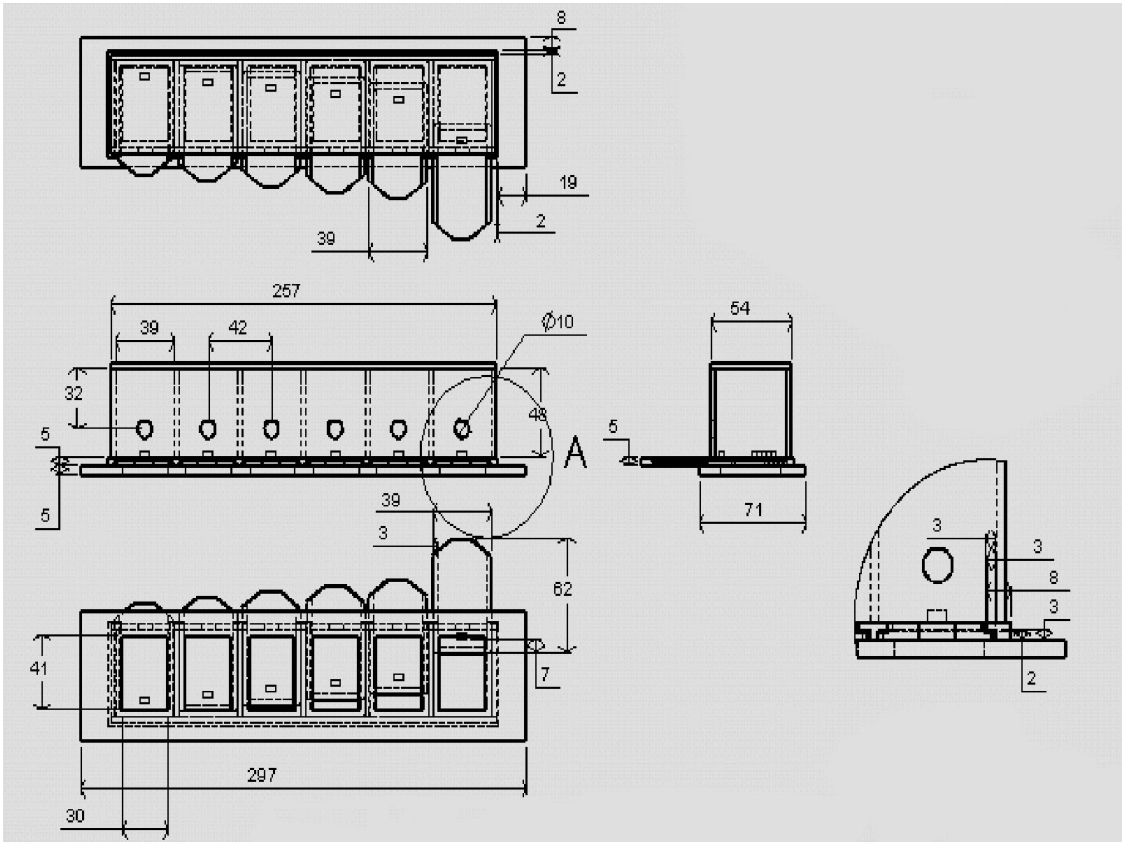

Fig. 1. Modified 6-celled Klun & Debboun module design. All measurements are in centimeters.

tained at the Walter Reed Army Institute of Research, Silver Spring, MD. The insects were reared (Gerberg et al. 1994) by feeding larvae ground tropical fish flakes (Tetramin Tropical Fish Flakes, Tetra Sales, Blacksburg, VA, www.tetra-fish.com). Adult mosquitoes were held at 12:12 (light:dark) h photoperiod at 27°C and 80% relative humidity with cotton pad moistened with 10% aqueous sucrose solution. Mated nulliparous *Ae. aegypti* and *An. stephensi* females (7–15 days old) were used in the testing. *Anopheles stephensi* had access only to water 24 h and *Ae. aegypti* had neither food nor water 24 h before testing.

Chemicals

Deet (N,N-diethyl-3-methylbenzamide) was obtained from Morflex, Inc. (Greensboro, NC) and Bayrepel [2-(2-hydroxyethyl)-1-piperidine carboxylic acid 1-methylpropyl ester] from Bayer Corporation (Bayer Consumer Care, Morristown, NJ). SS220 [(1S, 2′S)-methylpiperidinyl-3-cyclohexen-1-carboxamide] was synthesized earlier at the Chemicals Affecting Insect Behavior Laboratory (Klun et al. 2003). The chemicals were 98% pure chemically according to gas chromatographic analyses. Deet is a widely used repellent that is registered with the U.S. Environmental Protection Agency (EPA). Bayrepel is also registered with the EPA, and SS220 has been proven to be safe for use by humans (Snodgrass and Houpt 2002).

Bioassay methods

In vitro bioassays were conducted by using modified 6-celled Klune & Debboun (K&D) modules (Klun and Debboun 2000) (Fig. 1) and a specialized blood-feeding reservoir (Fig. 2). The module and reservoir were fabricated by Precision Plastics, Beltsville, MD, using Plexiglas. A constant-temperature water circulator (Lauda E100, Wobser GMBH and Co., Konigshofell, Germany) pumping at 15 L/min warmed the blood reservoir to 38°C. The K&D module for in vitro use was constructed with a flat base, whereas the in vivo K&D has a concave base that is designed to complement the curvature of a human thigh. Tests were conducted in the laboratory in front of a chemical fume hood. The blood-feeding unit was equipped with 6 reservoirs designed to match the sliding doors of the K&D modules. The 6 reservoirs were each filled with 6 ml outdated packed human red blood cells

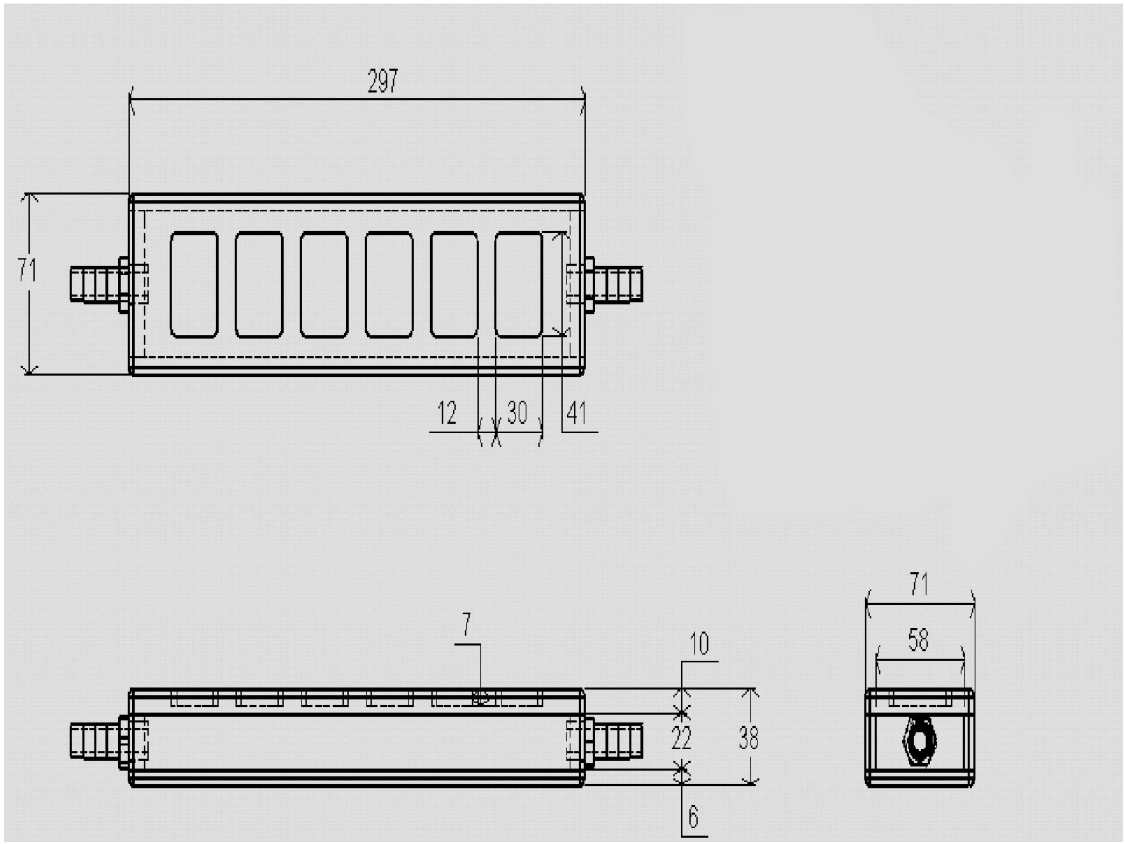

Fig. 2. Blood-feeding reservoir design. All measurements are in centimeters.

(Blood Services, Department of Pathology and Areas Laboratory Services, Washington, DC) supplemented with adenosine triphosphate (Sigma Chemical Co., St. Louis, MO) at a dose of 144 mg per 50 ml blood (Rutledge et al. 1976). Commercial baudruche (Joseph Long Inc., Belleville, NJ) and Edicol collagen film (Devro, Sandy Run, SC) membranes were used. Membranes were placed over the blood-filled wells and secured to the blood reservoir with a light coat of high-vacuum grease (Dow Corning Corp., Midland, MI). The membranes were replaced after each replicate observation and additional blood was added as needed. Each blood-feeding unit and blood was used for 6 replicates.

Areas of nylon organdy cloth (G Street Fabrics, Rockville, MD) corresponding to the six 4- × 3-cm openings of the K&D module were outlined on a ca. 29.7-cm × 7.1-cm cloth strip using a 6-celled marking template and an ink pen. As many as 6 marked rectangular areas were randomly assigned treatment code numbers and the treatment set represented a randomized complete block. Treatments were applied to cloths in a chemical fume hood. Ethanol solutions of each treatment (110 μl) or ethanol alone (control) were applied uniformly to each of the marked cloth areas with a pipette. Cloth, suspended horizontally over a tray using clips attached to tray ends, was always treated 0.5 cm outside the 4- × 3-cm outlines, resulting in ca. 20 cm^2 of treated surface. Cloth was dried thoroughly in the hood and then placed over the membrane that covered the blood.

A 29.7-cm × 7.1-cm × 0.40-cm Teflon® separator having 6 rectangular openings like the K&D module was positioned over the treated cloth. The function of the separator was to prevent contact of the module with treated cloth and thereby prevent contamination of the module with test compounds. A K&D module holding 5 mosquitoes in each of the adjacent module cells was positioned over the Teflon separator (Fig. 3) and the mosquitoes were exposed to the treatments for 3 min by opening the module's sliding doors. Most often, testing involved simultaneous use of 2 sets of 2 units connected in series to a single water-bath pump (2 replicates). The number of mosquitoes biting (proboscis inserted through the cloth and/or observed blood engorged) within each cell in the 3-min exposure was recorded and module doors were closed. Mosquitoes were used once in a test and

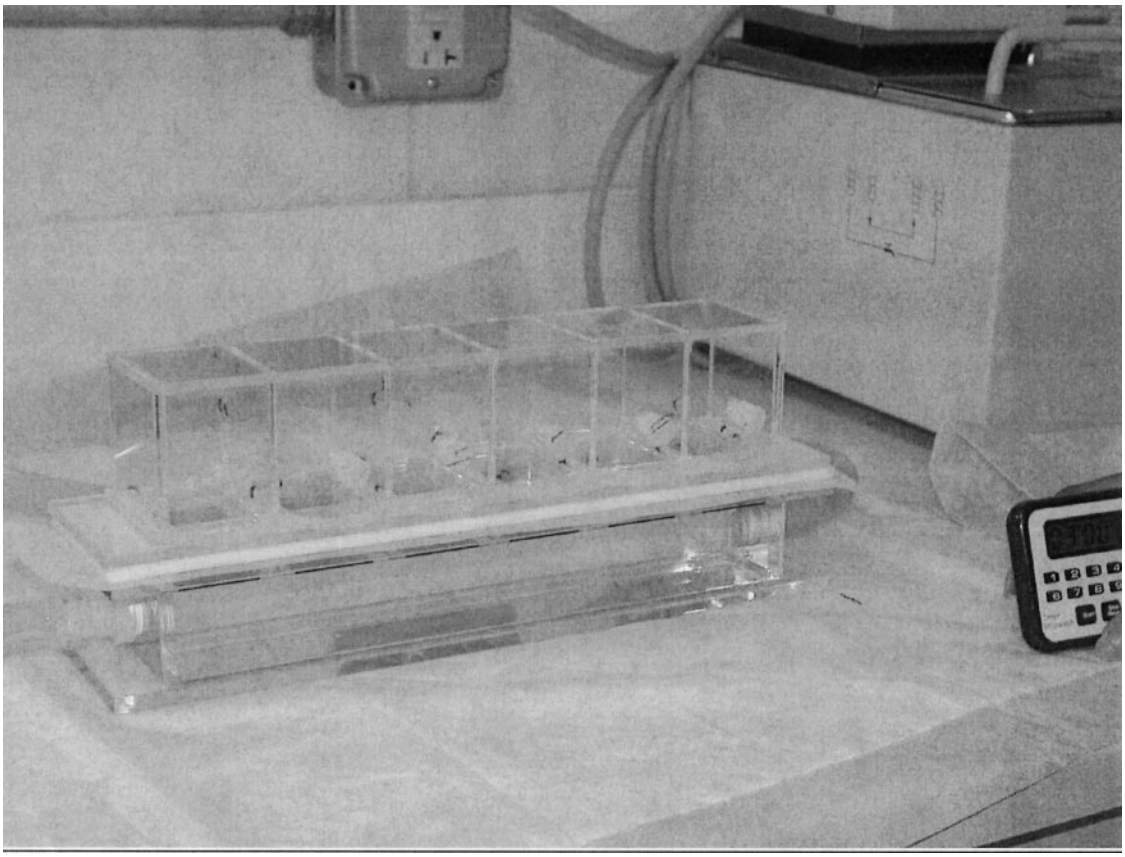

Fig. 3. Assembled bioassay unit showing the in vitro Klun & Debboun module position on a Teflon separator over a membrane-blood reservoir warmed to 38°C by a water-bath pump.

then frozen. In vivo human tests were conducted as described by Klun and Debboun (2000). The research involved 3 experiments.

Experiment 1. The biting responses of *Ae. aegypti* were measured against SS220, Deet, and Bayrepel at doses 0, 3, 6, 12, 24, and 48 nmol/cm^2 cloth using ethanol solutions and Baudruche membranes over the blood reservoirs. The dose–response experiment was procedurally identical to a test of the compounds against *Ae. aegypti* conducted earlier using human volunteers by Klun et al. (2003), where 2,160 mosquitoes were tested. To fit lines to the 2 data sets, we first arcsine transformed the proportions (to satisfy the homogeneity of variances analysis-of-variance assumption) and fit linear mixed models, where the arcsine transformed proportions depend on dose, compound, dose–compound interaction, and, for in vivo results, volunteer (modeled as a draw from a normal distribution with mean zero and variance estimated by the statistical software). The models were estimated using Proc Mixed in SAS.

Because the in vivo data were collected earlier than the in vitro data and mosquito-biting propensities can vary over time, we felt that, despite similar results for the 2 systems, the absolute biting rates were probably not comparable. An example of what could go wrong is the following. If mosquitoes bite less frequently in the in vitro system, but biting frequency in this population increased over time, then the observed similarity would be fortuitous. Thus, to make statistically valid comparisons, we felt it necessary to sample from the same mosquito population at the same time for both systems and did so in the following 2 experiments.

Experiment 2: Control, SS220, Deet, and Bayrepel were concurrently (same day) tested at a fixed dose of 24 nmol/cm^2 skin in vitro on baudruche membranes (cow mesentery) and in vivo using human volunteers against both *Ae. aegypti* and *An. stephensi.* The 24 nmol/cm^2 skin was chosen as the standard dose because experiment 1 and previous repellent dose $\times$ response study (Klun et al. 2003) with Deet and SS220 showed that it consistently reduced mosquito biting by at least 80% relative to untreated skin and therefore was a reasonable dose to use when comparing repellent efficacy of different compounds. For each species–system combination, we used 360 mosquitoes (1,440 total for the experiment). An analysis of variance using the SAS

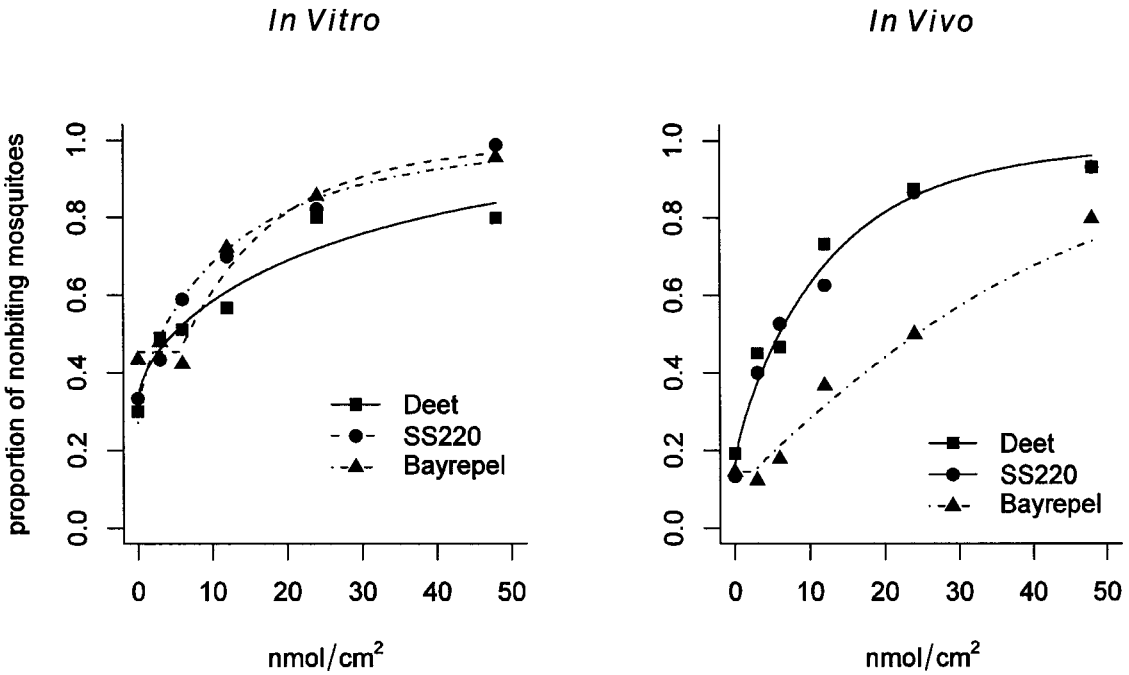

Fig. 4. Estimated in vitro and in vivo dose × response curves ($n = 18$) showing the proportion of *Aedes aegypti* not biting as a function of doses of Deet, SS220, and Bayrepel.

Glimmix macro (logistic regression for overdispersed binomial data and accounting for subject-to-subject variation) was conducted to test the effect of in vivo versus in vitro systems for significance, both as a simple main effect and as an interaction effect with compound. See Klun et al. (2004) for discussion of the effect of subject-to-subject variation in the analysis of human-derived (in vivo) data.

In the mixed models framework, logistic regression and fitting a linear model to arcsine-transformed proportions (where the proportion is the sum of binary responses divided by the number of trials) are alternative methods for handling a binary response variable (here, whether a mosquito bites or not). The former is theoretically more appealing but suffers somewhat in the approximations used in the statistical software; the latter is a variance-stabilizing approximation but then using well-accepted statistical models and mature software. In our experience, both methods produce similar results.

Experiment 3: The biting responses of *Ae. aegypti* and *An. stephensi* to control, SS220, Deet, and Bayrepel were concurrently (same day) tested in vivo using human volunteers at a fixed dose of 24 nmol/cm^2 skin and the same dose on cloth over Edicol membrane. We used a larger number (480 mosquitoes) for species–system combinations with *Ae. aegypti*; otherwise, methods and statistical analyses were the same as those used in experiment 2.

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.

RESULTS AND DISCUSSION

Experiment 1

While the magnitude of responses can differ among populations of the same species (Klun et al. 2004) and unpublished data suggest that there are temporal within-population changes in predisposition to biting, overall patterns should be consistent (Klun et al. 2003) and worth comparing. For both sets of data, Bayrepel appeared to have little or no effect at the lowest dosage; thus, we modeled this compound's effect at zero for low concentrations. The dose–response curves for Deet and SS220 were statistically indistinguishable for the in vivo data, and thus a single line was fit to these compounds. The slope parameter for Bayrepel was significantly lower ($P < 0.01$, $t = -4.36$, 20 df), indicating that Bayrepel was not as effective in the in vivo trials (see Klun et al. 2003 for a discussion of these results). For the in vitro system, none of the compounds were statistically distinguishable. Figure 4 shows results (data and fitted lines) from both systems back-transformed to the original scale (proportions of nonbiting mosquitoes) for display (hence the curvature in the lines). For the in vitro results, we have given each compound an individual best-fit line. The overall patterns of in vivo and

Table 1. Comparison of the mean proportion of *Aedes aegypti* and *Anopheles stephensi* not biting against a control and 3 repellents at 24 nmol compound/cm^2 cloth for in vivo bioassays and in vitro bioassays using Baudruche and Edicol membrane over blood.

		Mean proportion mosquitoes not biting			
Test type	Species	Control	Deet	SS220	Bayrepel
Baudruche membrane					
In vivo	*Ae. aegypti*	0.474	0.984	0.893	0.821
	An. stephensi	0.340	0.679	0.853	0.706
In vitro	*Ae. aegypti*	0.533	0.811	1.00	0.889
	An. stephensi	0.340	0.856	0.878	0.956
Edicol membrane					
In vivo	*Ae. aegypti*	0.534	0.984	0.968	0.912
	An. stephensi	0.460	0.767	0.825	0.697
In vitro	*Ae. aegypti*	0.558	0.808	0.992	0.833
	An. stephensi	0.433	0.722	0.922	0.933

in vitro results show a similar pattern of decreased biting with increased dose for all compounds, though separation of the compounds is better for the in vivo tests.

Experiments 2 and 3

The proportion biting for each species–treatment combination is given in Table 1. In the single-dose experiment, there were no statistical differences between the 2 membrane types or their interactions with compound for either of the 2 species (all $P > 0.05$). Thus, we collapsed over the membrane categories and tested, by species, a model containing the system and compound effects, their interaction, and subject (volunteer) effects. We found no overall difference in the biting rate between the 2 systems ($P > 0.05$ for both species). We would have detected a significant difference in overall biting rate if the 2 systems differed by as little as 9.3% (based on altering the scores from the in vivo system). We found large overall differences in biting rate among the compounds, due largely to the much higher biting rate for controls than for any of the repellent treatments ($P < 0.0001$ for both species). The interaction between compound and system was significant for both species, though in different ways. For *Ae. aegypti,* the 2 systems differed for Deet and SS220. In the in vivo system, the efficacy of the compounds was about the same (consistent with earlier results, e.g., Klun et al. 2003) in the in vitro system and SS220 tested superior to Deet. For *An. stephensi,* Bayrepel appeared to be more effective in the in vitro system than in the in vivo system. Otherwise, the results were very similar. Given the ease of conducting in vitro trials, our results suggest that this system is adequate for screening compounds for repellent activity, by comparing them with a well-established repellent, such as Deet. The moderately differential results seen from in vivo to in vitro assay modes with different compounds and mosquito species is reflective of the complex and interactive stimuli that must influence mosquito biting behavior. With this in mind, we are gratified and somewhat surprised that our new in vitro system correlates as well as it does with in vivo human-assay results.

We have empirically determined that, when 2 sets of 2 in vitro units (Fig. 3) are attached in series to 2 warming water pumps, it is possible to screen at least 100 candidate repellent compounds per 5-day week with 12 replicates/compound. Screening at this rate requires 9,000 female mosquitoes/wk. Thus, this new in vitro blood-feeding membrane system shows merit for high-throughput screening of new candidate compounds for their use as topical repellents for protection of humans. Promising compounds identified in the screening can be toxicologically evaluated and, if found safe, they would be ultimately tested on humans. For future screening tests, we intend to routinely use the Edicol membrane because it is comparatively inexpensive, synthetically prepared, and readily available.

Inasmuch as we found some differences between the in vivo and in vitro results (better compound separation and a different efficacy ranking in the in vivo system), final conclusions on a compound's utility should be drawn using in vivo test results. However, the substantial differences in biting rates between the controls and any of the repellents at moderate application rates, using either species, demonstrates that the membrane-based system will be a useful tool for identifying efficacious repellent compounds cheaply and quickly.

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