Prolonged Efficacy of IR3535 Repellents Against Mosquitoes and Blacklegged Ticks in North America

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ABSTRACT Here I report the first findings of consistently high, long-duration efficacy of IR3535 (ethyl butyl acetyl aminopropionate) formulations in the United States. I tested novel, controlled-release formulations of IR3535, at 10% in lotion and at 20% in pump spray and aerosol, against mosquitoes in the field and blacklegged ticks in the laboratory. These were also the first studies to be conducted under the authority of the U.S. Environmental Protection Agency's Human Studies Rule of 2006, and the protocols underwent science and ethics reviews by five entities. IR3535 is better known in Europe than in North America, having been marketed in the United States only more recently, and there are comparatively few publications on its efficacy. I began with pretrial studies of dosing behavior to compute formula-specific mean dosing rates for the subsequent efficacy trials. Dosing rates were lower than the 1 ml/600 cm rate commonly used to quantify efficacy. Complete protection times ranged from 7.1 to 10.3 h for mosquitoes and from 9.1 to 12.2 h for blacklegged ticks. Long protection duration resulted in many cases being truncated by darkness or eventual subject withdrawal, which suggests that actual protection times were probably greater.

KEY WORDS dosage, insect repellent, IR3535

Public concern about diseases transmitted by ticks and mosquitoes in the United States has increased conspicuously during the Lyme disease and West Nile virus epidemics (CDC 2005). A commonly advocated approach for preventing arthropod attack is "personal protection," a suite of avoidance techniques that includes the use of topical (skin) repellents (Barnard 2000). With the recent evolution of pyrethroid resistance in African anophelines (Guessan et al. 2006), the prospect of reduced efficacy of insecticide-treated bed nets for preventing disease highlights the importance of strengthening the available arsenal of personal protection measures for public health reasons.

IR3535insect repellent (ethyl butyl acetyl aminopropionate) is a synthetic molecule derived from the amino acid beta-alanine. It was designed in the early 1970s, has been marketed in Europe since the mid-1970s, and is currently available worldwide in >150 products (Puccetti 2006). IR3535 seems to have an unblemished safety record. It was introduced to the U.S. market in 1999, and it has achieved commercial success in Avon Products (Suffern, NY) repellents and has also recently been marketed in Sawyer Products (Safety Harbor, FL), and Chattem (Chattanooga, TN) repellent products. However, compared with N,N-diethyl-3-methylbenzamide (DEET), there are few studies measuring IR3535's efficacy (≈10 published studies with original data for mosquitoes and 2 for ticks).

A repellent's efficacy is most commonly measured by how long it protects at a certain level, under specified minimum conditions of arthropod pressure. I used a traditional protection threshold, complete protection time (CPT), scored as the time between a repellent's application and the first confirmed failure (FCF). A confirmed failure is a bite or landing (mosquito) or crossing (tick) that is followed by another such event within 30 min. The identical first confirmed bite (FCB) criterion is especially common in mosquito studies. Requiring a confirming failure is liberal in that it discounts observations of bites or crossings rare enough to fall outside of the arbitrary 30-min frequency criterion. Indeed, a minority of studies instead simply records the time until the first failure (with no confirming failure needed). However, the FCF criterion potentially yields a clearer indication of a systematic breakdown in protection. Requiring such redundancy to ascribe breakdown may be important because efficacy studies are commonly conducted with just a few human subjects, and individual subjects have a strong influence on repellent performance (Carroll 2006).

Compared with DEET and other proven repellent active ingredients such as Picaridin and p-menthane 3,8-diol (PMD; or oil of lemon eucalyptus), IR3535's reported performance to date is perhaps best described as moderate. For example, in a laboratory

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study against *Aedes*, *Culex*, and *Aedes* mosquitoes, five subjects were protected for an average of 3.2 h by 7.5% IR3535 compared with 4.8 h for 7.0% DEET and 7.6 h for 19.5% PMD (Barnard and Xue 2004; see Carroll 2006 for a discussion of concentration issues). Similarly, in a field study of repellents at similar concentrations (19.5–25%) against black salt marsh mosquitoes (*Ae. taeniorhynchus* Wiedemann), biting control subjects at an average rate of 19.5 ± 13.7 bites per min, Barnard et al. (2002) reported that Picaridin and DEET seemed to be the most repellent, followed by PMD and then IR3535 (in ethanol).

Beyond small sample size issues, the main limits to understanding IR3535's potential relate more to the paucity of studies either at higher concentrations or using contemporary formulation technology (Carroll 2006). Laboratory studies of 20% formulations by Thavara et al. (2001), Cilek (2002), and Leibisch (unpublished data; summarized by Puccetti 2006) reported that IR3535 and DEET perform similarly, with most durations in the 5- to 7-h range.

The studies reported here were commissioned by EMD Chemicals, a division of Merck KGaA (Darmstadt, Germany), to measure the mosquito and tick repellency of new IR3535 formulations, for purposes of registration with the U.S. Environmental Protection Agency (EPA). IR3535 itself is registered as a "biochemical" by the EPA.

Human Subject Ethical Oversight. The study protocol and informed-consent document were reviewed and approved by five entities: the Independent Institutional Review Board of Plantation, FL (a private institutional review board [IRB]), the California Department of Pesticide Regulation and Office of Worker Health and Safety, the U.S. EPA Office of Pesticide Programs, and its Human Studies Review Board (as mandated by 40 CFR parts 9 and 26; EPA 2006a).

Materials and Methods

Several aspects of these studies were influenced by the EPA Human Studies Rule (EPA 2006a). These include implementation of product-specific dosimetry, limitations to the number of control subjects and their exposure during the measurement of ambient biting pressure, and the elimination of a positive control. These aspects are elaborated where appropriate.

Human Study Subjects. Twelve human subjects were used to measure self-dosing behavior. Ten human subjects exposed each test material to arthropods for efficacy evaluation. A sample size of 10 was chosen for efficacy testing to give a reasonably large sample while avoiding exposing too many individuals to the minor risks associated with their participation. Subjects were 19–44 yr old, had not used repellents in the week before enrolling in the study, were not students or employees of the Study Director, and reported themselves in good physical condition, without allergy to mosquito bites. Subjects refrained from applying fragrances, drinking alcoholic beverages, or smoking beginning ≈12 h before testing began. All subjects read and signed the IRB-approved informed consent forms. Females were negative in pregnancy tests conducted the morning of the day they participated in testing and stated that they were not lactating.

Dosage Determination. Dosimetry was used to generate a mean application rate per square centimeter of limb surface, representing average consumer dosing behavior, that we used in applications to all subjects for efficacy testing. It was conducted at the Arthropod Behavior Laboratory at Carroll-Loye Biological Research in Davis, CA, over 3 d in October 2006. To calculate limb surface area, I multiplied limb length (wrist to elbow for forearms, ankle to knee for legs) by the mean of a set of four evenly spaced circumferences taken from across the entire length of each limb region. There were seven female and five male subjects.

In determining dosing rates for each repellent (lotion, pump spray, and aerosol), subjects were first instructed to practice delivering each from its dispensing container to establish their preferred method for efficiently achieving "full coverage" of all four lower limbs. I defined full coverage as a continuous and complete layer of the test material. Once each had established his or her preferred method, he or she conducted a series of three self-application replicates to each limb. Limbs were washed with soap and water, rinsed with 35% ethanol, and towel-dried before and after each application. Applications were made outdoors, immediately adjacent to the laboratory, at wind speeds <8 kph.

Before and after each application, the repellent container was weighed on a traceably calibrated Sartorius GC 2502 microbalance (Sartorius Group, Goettingen, Germany) (measurement increment, 0.001 g; 500-g capacity). For lotion, that measurement was sufficient to calculate the grams applied per unit surface area for each subject for each limb, with the subject mean taken from the three replicates.

For pump spray and aerosol, in contrast, I applied four new gauze "bracelet" dosimeters (2.52-cm-wide strips of Co-Flex cohesive flexible bandage) evenly spaced across the treated area in each replicate (at the sites on the limb at which the circumference measures were made). Before and ~1 min after each application, each set of four dosimeters was weighed on a traceably calibrated Sartorius H51 balance (measurement increment, 0.0001 g; 30-g capacity).

With this method, three values of weight loss (lotion container) or gain (pump spray and aerosol dosimeters) were recorded for each subject limb for each repellent. A mean subject dosage weight was calculated for each repellent based on the weight changes, in the case of pump spray and aerosol, multiplied by the quotient of the limb surface area divided by the dosimeter surface area. Dosage weight divided by limb surface area yielded a dosing rate in grams per square centimeter for each limb; the average arm and the average leg dosing rates were calculated for each subject. The single grand mean of these subject means for each repellent was used as the dosing rate for all subjects in efficacy testing. Those applications were

made volumetrically, based on the limb surface areas of each subject and the specific gravity of each repellent (lotion, 0.99 g/ml; pump spray, 0.95 g/ml; aerosol, 0.94 g/ml).

Mosquitoes and Study Areas. Mosquitoes were engaged as encountered in nature. Choice of field sites for efficacy testing was limited to sites with active nuisance-mosquito populations, but from which West Nile virus (WNV) or related viruses had not been isolated in the preceding month by the California State Department of Health Services.

In accordance with U.S. EPA guidelines, I tested each repellent in two habitat types. Field work was conducted between 25 October and 19 November 2006. I tested lotion and pump spray in a native forest in Butte County and a marsh in Glenn County, and aerosol in the Butte County forest and in a Merced County grassland with flowering native shrubs in which mosquitoes were sheltering and nectar-feeding. These habitats differed in the composition and relative abundance of foraging mosquito species present, although *Aedes melanimon* predominated in all of them.

Counties in California's Central Valley generally sustain large populations of mosquitoes late in the year, making the valley one of the only areas in the United States suitable for mosquito efficacy testing in autumn. At the same time, incidence of encephalopathic viruses in this season generally declines to zero. No sentinel chicken flocks recorded WNV after September in Merced County. One sentinel chicken flock had a single positive for WNV in the Butte County region in the month preceding the work, but flocks much closer to the sites had not. Importantly, a mid-October survey of several thousand mosquitoes in areas close to the Butte County site showed no presence of WNV in tests conducted by the University of California, Davis Center for Vector-borne Diseases (CDPH 2006).

Repellents and Their Application. Individual doses were prepared for each subject on the basis of the surface area of their limbs, measured as described above. Treatments were applied onto lower arms and legs (lotion and pump spray) or to lower legs alone (aerosol). When a subject served in more than one trial, a different limb was treated in the subsequent trial (with one inconsequential exception; see Discussion). Based on the results of the dosimetry analysis (see Results), the dosing rate was 0.001 ml of lotion and aerosol and 0.0006 ml of pump spray per square centimeter of skin surface.

Before a repellent was applied, subjects washed their limbs with a fragrance-free cleanser, rinsed them first with tap water and then with 35% ethanol in water, and dried them with clean towels. They donned white Tyvek coveralls (DuPont & Co., Wilmington DE), rolling sleeves or legs to permit repellent application. The rolled material was held in place with Co-Flex bandage (Andover Coated Products, Salisbury MA). The bandage was also wrapped to protect the vulnerable knee portions of the exposed limb from mosquitoes. Untreated control subjects followed the same preparatory regimen.

The test repellent was applied to treated subjects by Carroll-Loye technicians, using 1-ml syringes (0.01-ml measurement increment) and fingertips in surgical gloves, to spread the materials as evenly as possible. Aerosol was sprayed into a temporary container before being used to fill the syringe (permitting the propellant to evaporate). The sex distribution of treated participants was as follows: lotion and pump spray—forest site, seven females, three males; marsh site, four females, six males; aerosol—marsh site, three females, seven males; grassland site, two females, eight males. Once treated, subjects were instructed and frequently reminded to minimize abrasion of the treated skin by keeping it from contact with other surfaces as much as possible.

Exposure to Mosquitoes. All subjects wore head nets and surgical gloves in addition to Tyvek coveralls, and each carried a battery-powered mechanical aspirator (Hauscherr's Machine, Toms River, NJ). Treated subjects were partnered into groups of two. Each member of a partner pair was instructed to monitor their own exposed limb and that of their partner for mosquito landings during 1-min periods of exposure to mosquitoes (a "buddy system"). Partners stood ≈1 m apart, with pairs a minimum of 2 m from others. Likewise, untreated subjects were at least 2 m from other individuals. Exposures took place at 15-min intervals (based on exposure maxima negotiated with U.S. and California EPAs).

Mosquitoes at each site were distributed at adequate densities over extensive areas (more than ≈0.5 ha). Pairs were arrayed within such areas, at appropriate interpersonal distances but in no particular order, in advance of each interval. A technician advised subjects when each 1-min period began and ended. Subjects immediately exposed their limbs by drawing back the fabric of their coverall at the beginning of each exposure period. Subjects immediately aspirated (with a mechanical aspirator) any LIBing mosquitoes ("landing with intent to bite," defined as a stationary mosquito, initiating placement of proboscis on the skin) from the skin. All LIBes were reported to technical personnel, who recorded the events by subject code and the clock time of the exposure interval.

Subjects immediately covered exposed skin with the protective garment if a LIBe followed another in the same or in either of the two previous exposure periods. Subjects and data-recording personnel both monitored the occurrence pattern of LIBes to ensure proper protective responses.

U.S. EPA standards for efficacy testing require a minimum ambient biting pressure of approximately one landing per minute of a foraging mosquito on an untreated forearm or lower leg. In practice, achieving that rate normally requires that a substantial number of mosquitoes approach the subject during the exposure period, with some landing on other parts of the body as well (i.e., at a rate that would amount to substantial nuisance biting for an unprotected person). I measured ambient LIBe pressure with two untreated subjects who were exposed to mosquitoes on the same schedule as the treated subjects. These

two were experienced in efficacy testing and specifically chosen for this role because that experience reduced their likelihood of failing to respond properly to landing mosquitoes. Each was attended by two assistants with mechanical aspirators who also watched for mosquitoes and assisted in quickly removing them.

Each control protected the exposed limb with their Tyvek coverall as soon as the first LIBe occurred on it; accordingly, in most exposure periods, untreated limbs were exposed for <1 min (often ≈5–30 s). In addition, more than one mosquito sometimes landed on an exposed untreated limb, although that was not recorded, yielding records of presence (1) and absence (0) values. I report the sufficiency of ambient pressure during the tests as the frequency of exposures with at least one LIBe.

For protection, at the end of each 1-min exposure period, treated subjects carefully and quickly covered the exposed limbs with the coverall sleeve or leg (first releasing the elastic cohesive bandage strap holding the that part of the garment in place). Subjects were fitted in Tyvek suits large enough to provide ample room to minimize contact between the treated skin and the covering fabric. In addition, to reduce the possibility of uncontrolled abrasion or absorption of the test materials by the fabric, in the intervals between some exposure periods, subjects were able to move away from areas with mosquito activity and into a protective screen house (when the screen house was nearby). During those intervals, subjects were permitted to leave limbs uncovered. In the subsequent exposures, such subjects were instructed to closely monitor their exposed limbs while moving into position for efficacy testing. Limb exposure for untreated individuals in such cases remained as described (covered until the initiation of each exposure interval).

In the first tests (lotion and pump spray in the forest), I applied the test materials in the field, with first exposures ensuing in 15 min. Because of the long average duration of repellency measured in those tests, and the short autumnal daylength that truncated observations, in the other, subsequent trials, I applied the test materials in the laboratory, before driving to the field sites, resulting in exposure delays of 2–3.25 h. In those cases, times until the first landings were several hours, indicating that I did not artificially inflate protection times because of those delays.

A small proportion (totaling ≈1,400 specimens) of mosquitoes that landed on the protective Tyvek coveralls worn by subjects, or on exposed limbs, were aspirated, pooled by exposure interval, frozen, and later identified in the laboratory using taxonomic keys and stereomicroscopy.

Exposure to Blacklegged Ticks. I tested the repellents against nymphal blacklegged ticks (*Ixodes scapularis* Say). Ticks of this species and life stage are important in transmitting the Lyme disease pathogen to humans and pets. They may obtain the spirochete during larval feeding on wildlife, and pass it to human or pets as nymphs because their small body size makes them difficult to detect.

This study did not test tick-biting, and the risk of disease transmission during its conduct was judged to be extremely low. Nonetheless, to preclude the possibility of having infected ticks present, I used laboratory-reared, disease-free ticks. Nymphal blacklegged ticks were obtained from Dr. Thomas Mather of the University of Rhode Island Tick Laboratory. They were descended from field-caught adults from Rhode Island, and the population had been in the laboratory for several generations, obviating the risk of transovarial pathogen transmission. The ticks were reared on quarantined rodents at 23.5°C, >97% RH, and a 14-L:10-D light cycle. The hosts were screened to be pathogen-free for all tick-transmitted pathogens and hantavirus using appropriate culture, direct detection (polymerase chain reaction [PCR]) and immunological screening assays.

On receipt from Dr. Mather, the ticks were maintained and tested under slightly cooler and less humid conditions (see Results). These conditions maintained the ticks in a state of host-seeking readiness, so that their behavior would be comparable to that of ticks that people encounter in the natural environment. Nymphs were housed in plastic vials with a moist paper substrate. On test days, I placed vials of 50 fresh (unused) nymphs in small, water-filled trays from which ticks could not readily escape. Vial caps were removed. Subjects had practiced procuring ticks from the vials/trays with a small artist's paintbrush in advance of the test.

Repellents were judged on their ability to prevent ticks from walking ≥3 cm into the treated region of a forearm. Exposures began 15 min after the application of a test material. During each exposure period, ticks were first tested on the untreated arm to determine whether they were sufficiently active in questing. To assist subjects in positioning ticks and in determining how far ticks walked, after application of the test material, each subject was marked on the skin of both arms with three black dots with a Sharpie marker. One dot was placed on the palm, 3 cm distal to a second dot at the wrist (i.e., at the margin of the treated area), and the third dot was placed 3 cm into the treated area in a line toward the elbow.

Subjects worked in groups of four, initiating exposures at approximately the same time. To initiate an exposure, a subject used a paintbrush to lift a tick onto the palm dot of the untreated arm. To be included in the test, each tick needed to be active in locomotion and to travel as far as the third dot (6 cm toward the elbow) within 3 min of placement. Ticks usually began walking shortly after they were placed, and when necessary, the brush was used very gently to guide, but not push or force, a tick in the direction of the elbow. When used, such manipulation did not cause ticks to withdraw their limbs, drop from the subject, cease locomotion, or show other evident signs of stress. They were allowed to remain on the hand or arm for 3 min after moving in the direction of the elbow. Ticks meeting that criterion (all did; the distance required was only a small portion of their travel capacity in 3 min) were scored as "crossing on the untreated arm." They

were immediately transferred to the treated arm with the paintbrush for testing in like manner. Repulsion was scored when a tick changed its orientation away from, or parallel to, the margin of the treated area on approach or did not cross >3 cm toward the elbow within the 3 min allotted. Subjects occasionally retested repelled ticks by repositioning them with the brush near the treatment a second or third time within an exposure period to clearly satisfy the scoring criterion for repulsion. Ticks that crossed into the material for a distance of at least 3 cm toward the elbow (i.e., beyond the most proximal dot) were scored as "crossing" the treated arm.

Technical personnel monitored the subject practices during the test, and subjects were instructed to ask for assistance if uncertain about scoring any individual ticks; such crossings or repellencies were confirmed by the technicians. Subjects used a large, highly visible wall clock to measure time. Each tick was used in only a single exposure period on a single subject. Discarded ticks were placed in vials in trays labeled "used" and periodically were removed from the test area by technicians. Brushes were periodically replaced with new or cleaned ones. Brushes were cleaned in 50% ethanol and air-dried before reuse.

Exposures ended when a subject experienced a crossing in each of two, or in two of three, consecutive exposure periods (i.e., a confirming crossing). The first confirmed crossing (FCC) was defined as the first crossing in such a series. Subjects were withdrawn from further exposure when such an event occurred.

Environmental Conditions. Temperature, relative humidity, light intensity, and wind speed (field only) were recorded at ≈1-h intervals during all efficacy testing.

Data Analyses. Dosimetry data were entered into an Excel 2004 (Macintosh) spreadsheet for calculations of surface area and dosing means. Those means were double-checked with a handheld calculator. Dosimetry analyses, based mainly on subject means, consisted of nonparametric rank and correlation tests and parametric regression. These and other descriptive statistics were generated with SAS JMP software, version 5.0.1.2 (SAS Institute, Cary, NC).

I calculated CPT as the interval between application and the first confirmed LIBe or crossing (generically, FCF). The FCF was defined as the first failure followed by another within 0.5 h, i.e., in either of the subsequent two exposure periods. This measure is identical to that of FCB, which is commonly used in measures of repellency to biting insects, except that our practices minimized the probability that a subject was actually bitten by a foraging mosquito. CPT measured in this way gave a single duration value for each subject. Mean CPT was calculated across all 10 subjects per treatment and arthropod, and is presented herein with SD and 95% CI information.

In a number of cases, subject records were truncated by voluntary withdrawal (without failure) after many hours of testing or by darkness terminating a field trial. When that occurred, the overall duration of exposure was used to determine subject protection, with 15 min added when appropriate to estimate the minimum CPT obtainable (i.e., as if two failures had occurred during the subsequent one or two exposure periods).

Table 1. Dosing rate means ± SD (ml/cm^2) for each of the three test repellents[a]

Repellent formula	Mean arm	Mean leg
Lotion	1.16 ± 0.37	1.13 ± 0.48
Pump spray	0.71 ± 0.40	0.54 ± 0.35
Aerosol	1.42 ± 0.87	1.05 ± 0.60

[a] Twelve subjects applied each repellent three times to each of their four limbs, and a mean dose was calculated for each limb of each subject (48 means per repellent). Arm and leg means were averaged within subject, yielding one overall arm mean value and one overall leg mean value per subject (24 means). The 12 arm means and 12 leg means were averaged across subjects to produce the tabulated values for each repellent.

Test Results

Dosimetry. In aerosol and pump spray dosimetry, the small weight increment in the untreated control dosimeters (0.6–1.1% of the treated increment) was inconsequential and not considered further.

Test subjects varied up to about three-fold in the amount of lotion they applied, six-fold in the amount of pump spray, and seven-fold in the amount of aerosol. Dosing to left and right limbs was not fully uniform, but there were no systematic differences. The quantity of aerosol applied averaged about twice that of pump spray. Lotion was intermediate on arms, but on legs had the greatest mean application rate of the three formulations (Table 1). All of these rates are substantially lower than the prevailing standard of 1.67 ml/cm^2 (equivalent to 1.0 ml/600 cm^2), which would have been implemented in the absence of a dosing evaluation.

Individual subject doses for the subsequent efficacy trials were computed on the basis of limb surface area and the mean dosing rates of each formulation. Arms alone were used in tick testing, arms and legs for mosquito testing with lotion and pump spray, and legs alone for mosquito testing of aerosol.

Mosquitoes: Ambient Pressure. Untreated subjects exposed limbs for a maximum of 1 min during each exposure period (every 15 min). The arrival of a mosquito within any minute ended that exposure instantaneously, so no more than one mosquito was recorded during any such exposure (cases in which more than one mosquito appeared to land simultaneously were not distinguished). The resulting dataset shows the distribution of exposures with 0 or 1 LIBe on each of the two untreated subjects. Zero values were comparatively infrequent: across all six tests (three repellents, each in two habitats), LIBes failed to occur in from 3/29 (10%) to 1/32 (3%) of the exposures (on a per subject basis). Ambient pressure was therefore recorded as sufficient in 90+% of exposures. Note that mosquitoes were observed landing on the coveralls of both treated and untreated subjects

Table 2. Repellency against mosquitoes in nature: mean ± SD, range, and 95% CI of CPT[a] (h), the percentage of the 10 subjects that received a confirmed LIBe (CL[b]), and the mean ± SD no. of total LIBes per subject

Repellent	Mean CPT	Range	95% CI	%CL	Mean LIBes[c]
Lotion					
Marsh	8.5 ± 0.8	7.8–10	7.9–9.1	60	1.4 ± 1.3
Forest	7.3 ± 0.9	6.0–8.5	6.6–8.0	60	1.6 ± 1.3
Pump spray					
Marsh	8.4 ± 0.8	7.7–10	7.8–9.0	70	1.9 ± 1.3
Forest	7.1 ± 1.0	5.0–8.0	6.4–7.8	90	2.4 ± 0.8
Aerosol					
Grassland	10.3 ± 0.0	—	—	0	0
Forest	9.8 ± 0.3	8.8–9.8	9.5–9.9	10	0.4 ± 1.0

[a] CPT, the time until the first confirmed LIBe, or if none occurred, until 15 min after the conclusion of data collection, which would otherwise have been the earliest possible time for a confirming LIBe.
[b] A confirmed LIBe was defined as the first LIBe followed by another within 30 min (i.e., during one of the subsequent two exposure periods).
[c] Including the confirming LIBe if it occurred.

Table 3. Repellency against blacklegged ticks in the laboratory: mean ± SD, range, and 95% CI of CPT[a] (h), the percentage of the 10 subjects that received a confirmed crossings (CC[b]), and the mean no. of total crossings per subject

Repellent	Mean CPT	Range	95% CI	%CC	Mean crossings[c]
Lotion	9.1 ± 2.5	5.0–12.0	7.2–10.9	50	1.8 ± 1.5
Pump spray	12.2 ± 2.8	6.5–15.0	10.2–14.2	10	1.5 ± 1.3
Aerosol	11.0 ± 2.8	4.3–14.0	8.9–13.0	50	2.0 ± 1.9

[a] CPT, the time until the first confirmed crossing, or if none occurred, until 15 min after the conclusion of data collection, which would otherwise have been the earliest possible time for a confirming crossing.
[b] The proportion of subjects (of 10 total) experiencing a confirmed crossing, defined as the first crossing followed by another within 30 min (i.e., in one of the subsequent two exposure periods).
[c] Including the confirming crossing if it occurred.

in all exposure periods, so that there were no exposures in which mosquitoes were not approaching the treated subjects.

Repellency to Mosquitoes. Mosquitoes were strongly affected by all three IR3535 formulations and attempted to bite treated limbs in only a tiny minority of exposures. Table 2 shows the mean times between application and failure or withdrawal for each repellent in each of the study habitats. The comparatively long durations of protection, coupled with short autumnal daylength, meant that, in some cases, tests were truncated by darkness or subject withdrawal (because of exhaustion) rather than by product failure. In each test, there were no LIBes during the first several hours of exposure, even in those cases in which there was a 2- to 3-h travel delay between application and first exposure.

All three formulations protected subjects for an average of 7 h or longer. Particularly for lotion and aerosol, the truncation of exposures resulted in a reduction of the mean and the variance of the variables, because many to most subjects ceased exposures before receiving any LIBes. The 95% CIs regarding the mean CPTs are therefore quite narrow. LIBes, which were rare in most cases regardless of CPT, were highest in pump spray and lowest in aerosol.

Difference in performance among the formulations probably resulted in part from differences in IR3535 concentration and dosage. While, like aerosol, pump spray contained 20% IR3535 (versus 10% in lotion), pump spray dosing was substantially less than that of the others (Table 1). Aerosol, with the strongest combination of concentration and dosage, showed the best repellency. Across the two study habitats, 19 of 20 aerosol subjects were completely protected for the entire period of exposure. As a result, the CPT values underestimate the true protection times, perhaps substantially.

Mosquito Species. The efficacy trials were conducted on public lands managed for wildlife habitats that support large communities of mosquitoes. These communities remain reproductively active late into the year. In these field tests, the prominent species present were a combination of those most active under warm summer conditions (*Culex tarsalis* Coquillet, *Anopheles freeborni* Aitken), those active in summer and fall (*Ae. melanimon* Dyar, *Ae. vexans* Meigen), and those active mainly in cool months (*Culiseta incidens* Thompson). *Ae. melanimon* accounted for >90% of the mosquitoes collected in each habitat during the morning and early afternoon hours; the others became much more prominent in late afternoon, particularly *Cx. tarsalis*, *Cs. incidens*, and *Ae. nigromaculus* Ludlow. *Cx. erythrothorax* Dyar were also present in small numbers. All LIBes were by *Ae. melanimon*, despite numerous close approaches (e.g., touching arm or leg hair) by other genera during late afternoon exposures.

Field Environmental Conditions. These field studies were conducted under mild, humid conditions well suited for a repellent trial. Across all tests, temperatures ranged from 14 to 25°C, relative humidity from 24 to 91%, wind speed from 0.0 to 5.4 kph, and ambient light from 398 to 1,176 lux.

Ticks: Ambient Crossing Pressure. All ticks chosen by subjects were active in locomotion and met the questing criterion by traveling at least 6 cm toward the elbow on the untreated arm in 3 min.

Influence of Test Material on Probability of Crossing. Ticks were strongly affected by all three formulations, crossing in <5% of exposures (Table 3). Ticks that were repelled typically changed their trajectory on approach to the edge of the treated area of the arm, either reversing direction, or sometimes circumambulating the wrist near the treatment. Ticks that scored as crossing often remained in the treated area after crossing, failing to traverse to the elbow; instead, they ultimately reversed course or fell from the arm onto the laboratory bench.

As Table 3 shows, estimated CPTs across the repellents were 9 h or greater. Despite its low dosing rate, pump spray performed best against ticks, in apparent contrast to the results for mosquitoes. Pump spray subjects, by chance, remained in the test for a longer duration before withdrawing, but that trend is bal-

anced by the fact that only 1 of the 10 experienced a confirmed crossing. None of the formulations exhibited more than a 50% failure rate before subject withdrawal. All of those voluntary withdrawals were caused by the protracted nature of the test, which led to subjects' need to rest or turn to other activities.

The high frequency of subject withdrawals before failure indicates that the true test mean CPT was likely longer than estimated in each case.

Laboratory Environmental Conditions. Laboratory temperature ranged from 19 to 23°C, relative humidity from 41 to 51%, and ambient light from 119 to 401 lux.

Discussion

Dosimetry. This mosquito and tick repellent efficacy study was conducted as part of scientific characterization for U.S. EPA registration. This study was unusual among field repellent trials in that dosimetry studies were conducted in advance to determine typical user dosing behavior. The resulting average dosing rates for each product were used as the rates for all subjects in the efficacy trials.

Subjects varied substantially in their dosing behavior and dosing rates (Table 1). Subjects who applied the spray repellents more efficiently, with a greater proportion of the dispensed material reaching the limb, tended to deliver higher doses per unit area of their skin (S. P. Carroll, unpublished data). Despite individual variation in dosing rate, an advantage of applying the same, average dosing rate to all subjects was that it guarded against early failures of those who might otherwise "underdose" for the test conditions. Underdosing consumers might be expected to apply more repellent when protection is perceived as inadequate and perhaps to learn about adequate dosing from experience. That process cannot take place in standard repellent efficacy trials. Consequently, the average value from the dosimetry study was chosen as a reasonable approximation of sensible dosing behavior. Notably, that approach did not result in obvious overdosing of subjects. The mean dosages that emerged from the dosimetry analysis were substantially lower than those which would have resulted from common industry practice (e.g., 1 ml/600 cm^2, or 1 ml per forearm; ASTM 1994). Compared with that standard, rates were 25% lower for lotion, 7–32% lower for aerosol, and 55–65% lower for pump spray.

Mosquitoes. Ten percent IR3535 lotion and 20% IR3535 aerosol and pump spray provided consistent and prolonged protection (7 h or greater) against foraging mosquito communities in a series of natural habitats. Eight mosquito species were attracted to the test subjects, and those species included the three most important public health and nuisance genera, namely *Aedes*, *Culex*, and *Anopheles.* Like the West Nile vectoring *Culex* species, the highly anthropophilic *Culiseta incidens* was also present in large numbers in the grassland habitat near dusk, beginning ≈9.5 h after repellent application. In that aerosol test, all subjects were protected absolutely from LIBes until the cessation of exposures because of darkness at 1730 hours. In most cases, the duration of complete protection is likely substantially greater than the already striking values observed (see below). For all three formulations, subjects averaged very few LIBes (mostly well below two) for the entire period of exposure. This observation further indicates the test formulations' excellent repellency. In addition, the comparative safety of IR3535 (Puccetti 2006) suggests that reapplication is appropriate when using this repellent.

Exposure durations (1 min) and intervals (15 min) were designed to minimize risk to subjects while still providing sufficient exposure to generate a robust data set. The scientifically meritorious alternatives of having each subject serve as an internal control (by exposing an untreated and a treated limb simultaneously), or in a round-robin design in which each was untreated in at least one iteration, would have substantially increased biting risk. From an analytical standpoint, an additional scientific limitation of our approach is that ambient biting pressure was not directly quantifiable, so that the percentage of biting rate reduction cannot be calculated. Nonetheless, the regularity with which LIBes took place on control limbs during those brief exposures (90–97% of exposures, depending on the test day and untreated subject individual) indicates the consistently high rate at which mosquitoes approached subjects throughout each test day.

The ranges and 95% CIs for all three formulations are relatively narrow and comparable to those found for other long-lasting repellents (Schofield et al. 2007). However, the pervasiveness of truncated exposures (data-censoring) likely reduces the variability that we would have obtained, just as means would have risen, given longer exposures. Truncation also means that the distributions of CPT values was in many cases not well suited to parametric estimators such as the CI. Moreover, truncation was sufficiently common that even analyses designed to accommodate censoring (e.g., the Kaplan-Meier survival analysis) offered little additional information.

Truncated records mainly resulted from to a combination of two factors. First, the test materials protected longer than we anticipated based on published studies of other IR3535 formulations. Even more important, the months required to pass through the developmental U.S. EPA regulatory process meant that I tested later in the season, at a shorter photoperiod, than originally anticipated. Subjects nonetheless participated for many hours; the adaptation of treating before traveling to the field, implemented after the first test day, help to alleviate some of these difficulties.

Because of truncation, the data sets cannot comment with much power on statistical trends in product failure, but they instead give a rather robust and conservative estimate of the minimum duration of protection. The results suggest that consumers will likely be protected against a diversity of nuisance and disease-vectoring mosquito species for the full duration of many outdoor activities.

Ticks. All ticks crossed on untreated arms, indicating that they were suitably active for the efficacy trial. In the experimental setting, each of the test materials provided substantial and prolonged protection against blacklegged ticks. The average subject experienced two or fewer crossings over an average exposure period of ≈11 h. The best-protected subjects experienced no crossings in a 15-h sequence of exposures, whereas one more poorly protected subject experienced three crossings in 4.25 h. Note, however, that many ticks scored as crossing eventually dropped from the limb after climbing to the tips of arm hairs, rather than crossing completely through the treated area to reach the elbow.

Compared with mosquitoes, ticks are difficult to repel; products containing DEET or Picaridin (KBR 3023) sometimes offer only brief protection (Pretorius et al. 2003). Although few tests against ticks have been conducted with IR3535, their results are generally consistent with these findings of long-lasting, excellent repellency. For example, Cilek (2002) reported that IR3535 was more effective in repelling nymphal *Ix. scapularis* in laboratory trials than was DEET at comparable concentrations.

As in the mosquito trial results reported herein, a substantial proportion of samples were censored by subjects eventually needing to withdraw after many hours for practical reasons. Survival analysis again generated little additional information, but it should be remembered that the 95% CIs provided in Table 3, generated to meet EPA specifications, are for coarse comparative purposes only and are not based on parametric variables. With new awareness of the potential for such long-duration protection from ticks by IR3535, it would be straightforward to conduct future studies with volunteers prepared to participate for longer periods of time than was possible here.

Conclusions. The three IR3535 formulations provided unusually long-duration protection against mosquitoes and blacklegged ticks, even at comparatively low doses. Although I did not directly compare IR3535 to other active ingredients, the CPTs were close to those of the most effective DEET and Picaridin formulations against mosquitoes and potentially exceeded them against blacklegged ticks. Moreover, because of the long test durations, a large proportion of subjects withdrew from some tests before product failure, and the fall of darkness similarly limited the time span of data collection in many of the field tests (Tables 2 and 3). Both factors mean that true protection times were underestimated.

An analytical challenge that stems from the EPA requirements is that safety concerns resulted in the very limited exposure of untreated subjects to monitor ambient mosquito pressure. Rather than quantifying a rate over time, I was restricted to collecting and interpreting frequency data. My confidence that ambient pressure was sufficient was bolstered by the near perfect attendance of mosquitoes during untreated exposures, the large numbers of mosquitoes flying near and alighting on subjects throughout the test days, and the fact that EPA has successfully relied on the "one mosquito per minute" pressure threshold for a number of years. Improvements to this method might include measuring the number of seconds until a LIBe occurs in each exposure or carefully monitoring and counting all LIBes in a full 1-min exposure.

Some imperfectly controlled or unmeasured factors that could have conceivably influenced the results merit mention. For example, subjects were not perfectly balanced by sex; whereas there is some evidence of complex sex influences on repellence, detectable with very large sample sizes (reviewed by Carroll 2006), both sexes were nonetheless well represented in the tests. In addition, subjects treated on one limb on 1 d were sometimes treated on another limb for testing on the next day (with one exception of a subject inadvertently treated on the same limb). However, that practice is not likely to have influenced the results, because subjects washed treated limbs at least twice between tests with soap and 35% ethanol. Although I found no statistical evidence of an effect of prior treatment, the evident uniformity of the efficacy results across test days, subjects, and treatments makes that unsurprising. Other possible factors were the presence of a treated partner within about a meter of each treated subject (Barnard et al. 2002 found an influence of treated limbs on untreated limbs within subjects), and the likely loss of some test material to the Tyvek material of the coveralls. Although I cannot address these concerns directly, I think that they either did not have major effects of the results or are reasonably representative of common conditions of repellent use. Given that I did not aim to statistically compare the test materials to one another and that variables above are likely minor and of opposing influence, I do not regard them as impinging strongly on the basic efficacy results described. Last, further information would have been gained through the use of a positive control. Because during EPA review of the protocol (EPA 2006b), it was determined that several DEET-treated subjects were required to attain sufficient statistical power for comparisons, the sponsor withdrew the request because of uncertainty that the information to be gained, which was tangential to the study's chief objectives, merited exposing additional subjects to the test conditions. The performance of DEET repellents is well known, would likely have resembled, if not exceeded, that of the IR3535 test materials. The more interesting result would be the opposite, i.e., if IR3535 outperformed a DEET comparator, but that was not ascertained.

These findings contribute to the database suggesting a substantial increment in the efficacy of IR3535 when well formulated and at higher concentrations. From a user perspective, they complement the known excellent safety and cosmetic values of this active ingredient (Puccetti 2006). Because user acceptance is as critical as inherent repellency (Frances and Debboun 2006), IR3535 products at concentrations of 10% or greater should be seriously regarded as public health tools for protection from biting arthropods and the pathogens they transmit to humans.

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