EVALUATION OF N,N-DIETHYL-m-TOLUAMIDE (DEET) AS A TOPICAL AGENT FOR PREVENTING SKIN PENETRATION BY CERCARIAE OF SCHISTOSOMA MANSONI

BUZ SALAFSKY, KALYANASUNDARAM RAMASWAMY, YI-XUN HE, GARY L. ANDERSON, DIANE K. NOWICKI, AND TAKESHI SHIBUYA
Department of Biomedical Sciences, and Department of Pathology, College of Medicine, University of Illinois, Rockford, Illinois; Department of Pharmacology, Tokyo Medical College, Tokyo, Japan

Abstract. The effect of skin application of N,N-diethyl-m-toluamide (DEET) on the penetration and migration behavior of cercariae of Schistosoma mansoni was evaluated in vitro and in vivo in a mouse model. These studies showed that DEET at concentrations of 7.5% or higher was 100% effective in immobilizing and killing cercariae of S. mansoni in vitro. Ultrastructural studies on such DEET-exposed cercariae showed transformative and degenerative changes involving both tegument and deeper parenchymal structures. Fatal tissue lesions were evident as early as 5 min postexposure to DEET, and became more extensive with increasing exposure time. Cutaneous application of DEET (as a pure chemical in isopropanol or as a commercial insect repellent preparation) was more than 99% effective in preventing entry of S. mansoni cercariae into the mouse tail skin. Radiolabeling and tracer studies confirmed that 7.5% DEET applied to the skin prior to infection was highly effective in preventing schistosomal migration to the lungs.

Despite the stringent control and prevention methods that are being practiced, schistosomiasis continues to be a life-threatening infection in many tropical and subtropical parts of the world.1 In addition, an increase in the international travel in recent years has predisposed many travelers visiting such endemic areas to a greater risk of acquiring the disease and dissemination to new geographic locations.2 Schistosomiasis mansoni is a snail-transmitted water-borne disease.3 Skin is the only known route of entry for this parasite into humans and the infective stage (cercariae) can penetrate intact skin within minutes after water contact.1, 4, 5 Therefore, in endemic areas control measures are mainly targeted against 1) the snail intermediate hosts that transmit the disease, 2) cercarial skin penetration by use of chemical agents, and/or 3) mass therapy against established infections.1 Although reducing human exposure to water contact in endemic areas is probably the best way to control the infection, its implementation under field conditions is unrealistic.1, 2

During the past three decades several chemical compounds were screened for their ability to confer protection against cercarial penetration, especially after skin application. Among these, a few compounds such as N,N-diethyl-lauramide, niclosamide, Cederol® (Fluka Chemicals Corp., Ronkonkoma, NY), hexachlorophene, dibutyl phthalate, and benzyl benzoate were found to have significant cercaricidal effect in vitro.6–9 However, except for niclosamide, none of the other chemicals were tested for their clinical application under field conditions.10

During one of our routine studies to screen an appropriate vehicle for skin application of Cederol against cercariae, we accidentally found that a commercially available insect repellent (Off®; S. C. Johnson Inc., Racine, WI) was highly effective in preventing cercarial skin penetration. The active ingredient of this preparation was found to be 7.5% N,N-diethyl-m-toluamide (DEET). Published evidence show that DEET is probably one of the most widely used insect repellents worldwide because of its formidable effect against a number of arthropod pests.11, 12 However, there appear to be no studies that evaluated the ability of DEET in preventing cercarial penetration. To explore the potential effect of DEET as an anti-penetration agent against cercariae of Schistosoma mansoni, in this study we initially evaluated the in vitro effects of DEET on the morphology and viability of infective stages of the parasite. These studies suggested that DEET has a damaging effect on cercariae in vitro. We then analyzed the ability of topically applied DEET to confer protection against infections with S. mansoni in a mouse model.

MATERIALS AND METHODS

Parasite. Biomphalaria glabrata snails infected with S. mansoni were obtained from Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD). Cercariae were collected from infected snails as described previously.13

In vitro experiments. N,N-diethyl-m-toluamide (DEET) was obtained from Fluka Chemicals Corp. (Ronkonkoma, NY). Serial doubling dilutions of DEET ranging from 30% to 0.24% were prepared in isopropanol (Sigma, St. Louis, MO), and 50 μl of each dilution was transferred to individual wells of a 96-well, flat-bottom microtiter plate (Costar, Cambridge, MA). Control wells received 50 μl of isopropanol in each. The plates were then kept covered in a fume hood and the fluid was allowed to evaporate overnight. The following day approximately 50 cercariae of S. mansoni suspended in 50 μl of RPMI 1640 medium (pH 7.4; Gibco BRL, Gaithersburg, MD) was placed in each well and the viability was monitored at 30 min, 60 min, and 4 hr. A total of 17 replicas was performed for each dilution and each time point in any one given experiment. To determine the viability of cercariae, 10 μl of 0.05% neutral red (Sigma) was added to each well at the end of experiment and the percentage of live and dead cercariae was determined as described previously.14

Ultrastructural studies. The effect of DEET on cercarial morphology was evaluated by transmission electron microscopic studies. In these experiments, approximately 300–500 cercariae of S. mansoni suspended in 100 ml of RPMI 1640 medium was placed in each well of a 96-well plate coated with varying concentrations of DEET (7.5–40%) or isopropanol (control) as described above. Samples were then collected at 5, 10, 20, 30, and 240 min after exposure to the DEET and processed for transmission electron microscopy as described earlier.15 Briefly, cercariae collected from each well were transferred to a fresh tube containing RPMI 1640 medium plus 10% fetal calf serum and concentrated into a
showed that a 7.5% concentration of DEET immobilized cercariae. A time-lapse study confirmed that the cercariae were dead. When placed in these concentrations, cercariae were instantly immobilized. Neutral red staining 30 min later confirmed that the cercariae were dead. A time-lapse study showed that a 7.5% concentration of DEET immobilized the cercariae within 5 min and by 4 hr all the cercariae were dead. However, concentrations of less than 7.5% DEET was less effective in immobilizing or killing cercariae of *S. mansoni*.

**In vivo experiments.** Male mice of the CD1 strain weighing approximately 18–20 g, were obtained from Charles River Laboratories (Wilmington, MA) and used in these experiments. The use of animals in these experiments was in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals as stated in the National Institute of Health publication: Guide for the Care and Use of Laboratory Animals.

For skin application, mice were individually kept in restraining cages and their tails were dipped for 5 min in a DEET solution prepared in isopropanol. The tail skin of control animals was exposed similarly to isopropanol alone. Following application, the skin was air-dried for 30 min, rinsed in distilled water for 1 min, and exposed to 250–300 radiolabeled cercariae of *S. mansoni* suspended in 10 μl of distilled water. Sixty minutes after skin exposure to infection, the tails were air-dried for 30 min and mice were returned to their cages.

Radiolabeled cercariae were prepared as described earlier. Briefly, 20–30 *S. mansoni*-infected *B. glabrata* snails were exposed to tritiated methionine (25 mCi/ml; specific activity = 1,047 Ci/mmol; catalog # 51006; ICN Pharmaceuticals, Inc., Irvine, CA) for 24 hr at 27°C and radiolabeled cercariae were shed 72 hr later. This procedure consistently yielded > 99% radiolabeled cercariae.

To determine skin penetration and worm establishment, tail skin and lungs were collected on day seven after infection and processed for compressed organ and autoradiography techniques as described previously. Briefly, the tail skin and lungs were mounted on to a cardboard sheet, covered with plastic wrap, and pressed in a tissue press for 48 hr. The pressed tissue were then exposed to x-ray film (Fuji RX; Fisher Scientific, Pittsburgh, PA) at −70°C for 7–9 days. Following autoradiographic development, the spots (which represent reduced silver foci on film) were counted and percent worm establishment was calculated (number of spots/total number of cercariae used for infection × 100).

**Statistical analysis.** Statistical significance of the means of the values were tested by one-way analysis of variance using the Sigmastat program (Jandel Scientific, San Rafael, CA). Probability values of 1% or lower were considered to be significant. All values are expressed as the mean ± SD.

**RESULTS**

**In vitro cercaricidal effects of DEET.** In vitro experiments show that DEET at a concentration of 15% and higher was highly lethal to cercariae of *S. mansoni* (Figure 1). When placed in these concentrations, cercariae were instantaneously immobilized. Neutral red staining 30 min later confirmed that the cercariae were dead. A time-lapse study showed that a 7.5% concentration of DEET immobilized the cercariae within 5 min and by 4 hr all the cercariae were dead. However, concentrations of less than 7.5% DEET was less effective in immobilizing or killing cercariae of *S. mansoni*.

**Effects of DEET on the morphology of cercariae.** Control cercariae (Figure 2a and b) demonstrated intact, corrugated tegument with an external glycocalyx, single membrane, and an absence of cyton granules. The muscular layer and deeper parenchyma were intact, without edema or degenerative changes. Ultrastructural changes after exposure to DEET were of two types: transformative changes that mimicked normal changes in the tegument of cercaria during its transformation to schistosomulum, and degenerative changes indicative of severe cellular damage. Both changes were evident as early as 5 min postexposure, but became more severe with increased duration of exposure.

Typical changes after 5 min of exposure to DEET include cyton granule migration into the tegument (indicative of transformational changes), diminished quantity of glycocalyx, and early degenerative changes such as intra- and extracellular swelling, diffuse edematous changes in the parenchyma, and focal lysis (Figure 2c). These changes were noted in all the parasites examined, although to a varying degree. Changes progressed in degree at 10- and 30-min duration of exposure (Figure 3 and 4a). Transformative changes, with loss of glycocalyx and cyton granule migration, became more prominent, but were generally overshadowed by degenerative changes. Massive accumulation of extracellular fluid was common, causing a flattening of tegument, and focal breaches of the integrity of the tegument were observed. Intracellular degenerative changes of vacuolization, condensation, and disruption of myofilaments in the muscle layer, nuclear swelling and condensation of chromatin, and
Figure 2. Control cercaria of Schistosoma mansoni. a, survey of tegument and deeper structures. Note the intact, corrugated tegument (T), compact muscle layer (M), and deep parenchyma without cellular swelling or edema (P) (bar = 1 μm). b, intact tegument (T) with glycocalyx (G) overlaying intact muscle. Note the lack of cyton granules in the tegument (bar = 0.5 μm). c, ultrastructural changes five minutes after exposure to N,N-diethyl-m-toluamide. Note the cyton granule migration (arrow) and cellular swelling with nuclear chromatin clumping (S). The glycocalyx is present but diminished overall (bar = 1 μm).
Figure 3. a, ultrastructural changes in Schistosoma mansoni 10 min after exposure to N,N-diethyl-m-toluamide (DEET). Note that the tegument is flattened and there is extensive migration of cyton granules (C). Note the marked expansion of extracellular space (arrows) and condensation of muscle elements (M) (bar = 0.5 μm). b, tegument showing extensive vacuolization and breaches (arrows) at several points (bar = 0.5 μm). c, ultrastructural changes 30 min after exposure to DEET (bar = 0.5 μm). d, severe tegumental loss with exposed underlying tissue and surface blabbing (arrow). Deeper parenchyma showed condensation of nuclear chromatin (N) and evidence of cellular debris (double arrows) (bar = 1 μm). e, vacuolization (arrow) and degeneration of tegument (T) with disorganization of muscle layer (M) (bar = 0.5 μm).
FIGURE 4. a, ultrastructural changes 30 min after exposure to N,N-diethyl-m-toluamide (DEET) showing flattened tegument and severe degenerative changes of deep parenchyma with widespread cellular lysis (arrow) (bar = 1 μm). b, ultrastructural changes 240 min after exposure to DEET showing severe disruption of tegument and muscle layers. There is severe edema and nuclei are karyorrhectic (bar = 1 μm).
cellular lysis were progressive over time. Frank lysis of the cercariae was observed 240 min after exposure to DEET (Figure 4b).

**In vivo experiments.** The above *in vitro* experiments suggested that at concentrations of 7.5% or higher, DEET was highly effective in immobilizing or killing cercariae of *S. mansoni*. Therefore, we initially decided to evaluate the *in vivo* ability of the commercially available insect repellent Off® (with 7.5% DEET as the active ingredient) in preventing penetration of cercariae of *S. mansoni* into the mouse skin and its further migration to the lungs.

Results of these *in vivo* studies showed that in controls animals treated with isopropanol only, a significant proportion (more than 28%) of the cercariae penetrated intact tail skin. Of the total that penetrated the skin, more than 65% had migrated to the lungs by day seven. Application of the insect repellent containing DEET to the tail skin prior to infection, however, resulted in a significant decrease in worm establishment. When the experiments were repeated with 7.5% DEET in isopropanol, a similar result was obtained (Table 1). These results showed that application of 7.5% DEET to the tail skin prior to infection resulted in a 99.87 ± 0.13% decrease in skin penetration by cercariae of *S. mansoni*. Further analysis showed that the small percentage of cercariae (0.13%) that managed to enter the tail skin failed to reach the lungs (Table 1).

**DISCUSSION**

Since its first commercial application in 1956, DEET has become one of the most widely used insect repellents in both human and veterinary use. Repeated studies show that DEET is highly efficient in repelling a wide variety of arthropods, particularly with respect to mosquitoes, sand flies, and ticks given their major role in spreading diseases in both human and animals. In most cases, a single skin application of DEET was effective in repelling the insects for several hours. In this study, we show that application of DEET may also be used to prevent exposure to schistosomiasis. To our knowledge, this is the first time anybody has shown the potential use of DEET as a prophylactic agent in the control against schistosomiasis.

Results presented in this study show that DEET has a *cercarial* effect *in vitro*. At concentrations greater than 15%, DEET instantaneously immobilized cercariae of *S. mansoni* and killed them as shown by staining with neutral red. As has been observed with other toxic substances, the initial response of cercariae to DEET appeared to be a transformation behavior, as shown by the shedding of their tails and emptying of their acetabular gland contents. Ultrastructural changes observed 5 min after exposure to DEET were also supportive of the transformation behavior in that there was loss of glycocalyx, and migration of cytol granules into the tegument similar to those seen in the transformed schistosomulum.

Prolonged exposure of cercariae to DEET resulted in progressively severe structural changes to the tegument and internal organelles of the parasite. Disruption of the tegument, muscle, and deep parenchyma occurred with increasing frequency and severity. By 4 hr, nearly all of the internal structures had lost their normal architecture and cellular nuclei were karyorrhectic. *In vitro* experiments and staining with neutral red also confirmed these fatal lesions. When cercariae were exposed to DEET at concentrations of 7.5% or higher, there was 100% mortality of cercariae in the cultures. This suggested that DEET is highly toxic to cercariae of *S. mansoni*, especially at concentrations greater than 7.5%. However, at concentrations less than 7.5%, the damaging effects appeared to be minimal. This suggested that to have any protective effect, DEET should be used at concentrations greater than 7.5%.

Pharmacokinetic studies in laboratory animals showed that DEET is absorbed fairly quickly through skin, and metabolized completely with more than 78% excreted via urine over a period of several days. Similar studies in human also showed that DEET is absorbed through the skin. However, the skin absorption rate in humans was found to be comparatively low (approximately 8% in 2 hr). Although, in the present study we did not measure the time-dependent concentration kinetics of DEET in the skin, a slower rate of absorption from the skin might be beneficial in that it could prolong the bioavailability of DEET in the superficial layers of the skin. Thus, a single application of 7.5% DEET to the skin might effectively protect against cercarial penetration for some time. In our mouse studies, infections were given 30 min after a 5-min exposure to 7.5% DEET and all the mice were protected. The fact that mouse tails, following air-drying, were rinsed for 1 min in distilled water suggests some skin absorption of DEET may have occurred. It is not possible to predict the duration and extent of protection at this concentration under field conditions given the impact of a number of variables including water contact, sweat, etc.

N,N-diethyl-m-toluamide is generally considered safe when used at lower concentrations and in presence of appropriate vehicles. However, when used at very high concentrations, pure DEET solution can induce neurotoxic symptoms in some individuals. Several cases of neurotoxicity to DEET have been reported in both human and animals. In one of our studies, application of DEET to the abdominal skin at concentrations of ≥ 40% was found to induce neurotoxic symptoms in mice. These symptoms include ataxia, seizures, tremors, and death. However, when used at concentrations ≤ 20%, DEET had no obvious adverse affects in mice. Since the cercarial and anti-penetration effects of DEET were maximal at a concentration of 7.5%, there is a potential for the use of DEET as a safe prophylactic agent in the control of human schistosomiasis mansoni.

N,N-diethyl-m-toluamide may also be potentially useful in the control of cercarial dermatitis (i.e., swimmer’s itch) in humans that is associated with exposure to animal schistosome cercariae. Cercarial dermatitis is a common problem.
among individuals engaged in recreational water sports such as fishing, boating, rafting, and swimming.\textsuperscript{33, 34} Moreover, cercarial dermatitis is a recurrent problem for agricultural workers in many locales in which water contact is high, e.g., rice farming.\textsuperscript{15}

Next to malaria, schistosomiasis is the major life-threatening tropical disease of humans.\textsuperscript{1, 2} Nonimmune travelers visiting endemic areas are at a high risk of acquiring this disease and disseminating the parasite to nonendemic areas.\textsuperscript{2} Since skin is the only route of entry for this parasite into humans, interventions that prevent entry of cercariae into the skin should control the infection.\textsuperscript{4, 5} Results presented in this study are encouraging and further analysis needs to be done before field application of DEET is considered for the control of schistosomiasis.

Acknowledgments: We thank Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD) for providing the \textit{S. mansoni}-infected snails.

Financial support: This work was supported by funds from the Pfizer Foundation, Japan.

Authors' addresses: Buz Salafsky, Kalayan sundaram Ramaswamy, and Yi-Xun He, Department of Biomedical Sciences, College of Medicine, University of Illinois, 1601 Parkview Avenue, Rockford, IL 61107. Gary L. Anderson and Diane K. Nowicki, Department of Pathology, College of Medicine, University of Illinois, 1601 Parkview Avenue, Rockford, IL 61107. Takeshi Shibuya, Tokyo Medical College, Tokyo, Japan.

Reprint requests: Buz Salafsky, Department of Biomedical Sciences, College of Medicine, University of Illinois, 1601 Parkview Avenue, Rockford, IL 61107.

REFERENCES