



Research Section

Topical permethrin exposure inhibits antibody production and macrophage function in C57Bl/6N mice

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Abstract

Permethrin was applied to the shaved dorsal interscapular region of C57Bl/6N mice at doses of 0.5, 1.5 or 5.0 µl/day. These doses corresponded to approximately 22–220 mg/kg/day topical insecticide. Mice were exposed to permethrin in this manner daily for 10 or 30 consecutive days, or every other day for 7 or 14 exposures. The splenic macrophage chemiluminescent response was depressed in a dose-dependent manner at 2 and 10 days post-exposure to permethrin. Phagocytic ability of macrophages was not inhibited. Antibody production as shown by plaque-forming cell (PFC) assay decreased significantly after 10 consecutive days of exposure to permethrin. These data indicate that topical permethrin exposure may produce systemic immune effects. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Permethrin; Immunotoxicity; Macrophage; Chemiluminescent response

1. Introduction

Permethrin is a member of one of the newest classes of insecticides, the synthetic type I pyrethroids. This compound has gained in popularity due to its photostability, high activity against insects, and relatively low mammalian toxicity compared to other insecticide classes (Papadopoulou-Mourkidou, 1983). Military uniforms treated with permethrin were used during the Gulf War for prevention of insect-borne disease (e.g. leishmaniasis) (Lillie et al., 1988; Scholdt et al., 1989; Schreck and Kline, 1989; Schreck et al., 1986). Snodgrass (1992) calculated that topical exposure of permethrin to humans wearing such uniforms was about 34 µg/kg/day.

Abbreviations: Con A, Concanavalin A; HBSS, Hanks' balanced salt solution; IFN-γ, gamma interferon; IL-2, interleukin-2; LPS, lipopolysaccharide; NK, natural killer; PFC, plaque-forming cell; PHA, phytohaemagglutinine; PMA, phorbol-12-myristate 13-acetate; SRBC, sheep red blood cells; TNF-α, tumor necrosis factor alpha

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Veterans of the Persian Gulf War have reported symptoms that have collectively been referred to as "Persian Gulf Syndrome" (Klaustermeier et al., 1998; Proctor et al., 1998; Unwin et al., 1999). These included loss of memory, fatigue, muscle and joint pain, ataxia, skin rash, respiratory difficulties and gastrointestinal disturbances (Murphy et al., 1999). Doucet (1994) suggested that these symptoms may result, in part, from multiple assaults on the immune system. Subsequently, some immune parameters were found to be significantly different between war veterans reporting health effects and controls (Zhang et al., 1999). These alterations included increased T lymphocytes and decreased natural killer (NK) cells in peripheral blood, and increased serum interleukin-2 (IL-2), IL-10, gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α).

Immunotoxicity from permethrin-like compounds has been reported in laboratory animals. The type II pyrethroid cypermethrin caused decreased humoral and cellular immune responses in rats and rabbits (Desi et al., 1985; Madsen et al., 1996). Tamang et al. (1988) similarly observed suppressed cellular and humoral

immune responses in cypermethrin-treated mice and goats. Deltamethrin, a type I pyrethroid, inhibited humoral and cellular immune responses (Lukowicz-Ratajczak and Krechniak, 1992; Queiroz, 1993) and caused thymic atrophy in mice (Enan et al., 1996). Enan et al. (1996) also reported that deltamethrin induced apoptosis of thymocytes in treated animals via alteration of the Ca/CaM-dependent protein kinase-phosphatase cascade.

Studies evaluating immunotoxicity of permethrin are limited to a few reports. *In vitro*, permethrin inhibited the mitogenic response of murine lymphocytes to Concanavalin A (Con A), lipopolysaccharide (LPS) (Stelzer and Gordon, 1984), and phytohaemagglutinine (PHA), and decreased the production of IFN- γ and IL-4 (Diel et al., 1998). Mice exposed to permethrin by oral gavage displayed several impaired immune responses, including inhibited mixed lymphocyte responses and depressed cytotoxic T lymphocyte and NK cell activity (Blaylock et al., 1995).

Permethrin crossed the skin in rabbits (Snodgrass, 1992), pigs and rodents (Baynes et al., 1997), suggesting the possibility of systemic effects following topical exposure. We recently observed decreased thymic weight and persistent inhibition of the contact hypersensitivity response in mice dermally exposed to permethrin (Punareewattana et al., 2000). The present study extends these results to include inhibited splenic macrophage function [chemiluminescent response (H_2O_2 production)] and depressed humoral immune responses (antibody production) in mice after subacute topical exposure to permethrin.

2. Materials and methods

2.1. Mice

Female C57Bl/6N mice (21.5 ± 1.0 g; Harlan Sprague Dawley, Indianapolis, IN, USA) were used in these studies. Mice were quarantined for 1 wk prior to initiation of experiments. Following the quarantine period, mice were randomly assigned to treatment groups and housed one mouse per cage. Mice were anesthetized by brief inhalation exposure to methoxyflurane, and the interscapular area was shaved using electric clippers. Mice were maintained under controlled conditions of temperature ($22 \pm 1^\circ C$), humidity (40–60%), and lighting (12-hr light/dark cycle) and provided with food and water *ad lib.* throughout the course of the experiments. The mice were observed daily for clinical change.

2.2. Permethrin preparation and treatment protocols

Permethrin (91.6%) was provided by the U.S. Army Center for Health Promotion and Preventive Medicine

(CHPPM; Aberdeen Proving Ground, MD, USA). Mice were shaved in the interscapular region 2 days before dosing began. Mice dosed for 30 consecutive days or every other day for 28 days were shaved a second time on day 14 or 15. Mice were treated with permethrin using three dosing solutions defined as low-dose (0.5 ml permethrin in 4.5 ml corn oil), middle-dose (1.5 ml permethrin in 3.5 ml corn oil) and high-dose (5.0 ml permethrin). Corn oil was used as the control solution. In all cases, six mice were used per treatment group (i.e. $n = 6$). All dosing solutions were stored in the dark at room temperature. Mice were dosed by interscapular topical exposure with 5 μ l of respective dosing solutions using an Eppendorf micropipettor. After the 5 μ l dose was applied to the shaved skin, the smooth side of the pipette tip was used to spread the dose over the skin. Dosing was daily in this manner for 10 or 30 consecutive days, or every other day for seven or 14 exposures. This dosing schedule was selected to mimic human military exposure where permethrin-treated uniforms may be worn for several consecutive days or intermittently for extended periods. For descriptive purposes, the four dose groups were named as follows:

Exposure 1 = every day for 10 consecutive days

Exposure 2 = every other day for 7 exposures

Exposure 3 = every other day for 14 exposures

Exposure 4 = every day for 30 consecutive days

Mice were sacrificed by CO_2 inhalation on days 2, 10 or 30 after termination of dosing, weighed, and evaluated for immune effects of the chemical exposure.

2.3. Cell preparation for macrophage function

Spleens from each mouse were collected and placed separately in 2 ml of culture medium (RPMI 1640; Mediatech, Cellgro, Herndon, VA, USA) in a 60 \times 15 mm culture dish (Fisher Scientific, Norcross, GA, USA). Splenic cells were gently dissociated in the culture medium using a metallic sieve screen (Sigma Chemical Co., St Louis, MO, USA) and curved forceps. Erythrocytes were removed from splenic samples by suspending cells in lysing solution (0.015 M NH_4Cl , 1.0 mM $NaHCO_3$, 0.1 mM EDTA) for 5 min at room temperature. Cells were then washed twice in culture medium, resuspended in 2 ml standard buffer (Hanks' balanced salt solution; HBSS), and counted using a CASY-1 electronic cell counter (Scharfe System GmbH, Germany).

2.4. Chemiluminescence assay

The production of H_2O_2 in phorbol-12-myristate 13-acetate (PMA)-stimulated splenic macrophages was determined by the method of Bass et al. (1983). Briefly,

splenic cell suspensions (0.5×10^6 cells) were incubated with 5 μ l dichlorofluorescein-diacetate (DCF-DA; Molecular Probes, Eugene, OR, USA; 5 mM) for 15 min at room temperature. Following incubation with DCF-DA, cells were stimulated by the addition of 10 μ l PMA (Sigma; 100 ng/ml) in a subsequent 30-min incubation period. Cells were then placed on ice to stop the reaction and immediately analyzed by flow cytometry. Background fluorescence, determined using unstained cells from each treatment group, was subtracted from respective populations incubated with the fluorescent probe.

2.5. Phagocytosis of fluorescent microspheres

The phagocytic capacity of splenic macrophages was determined by a modification of the method of Dunn and Tylor (1981). Briefly, cells were aliquoted as above in polystyrene round-bottom tubes at 0.5×10^6 cells in 0.1 ml standard buffer. Fluoresbrite microspheres (1.16 μ m; Polysciences, Inc., Warrington, PA, USA) were washed twice in standard buffer, and then sonicated on ice (Ultrasonic Cell Disrupter, Misonix, Inc., Farmingdale, NY, USA) for 30 sec at 35% to disrupt aggregated microspheres. A 10 μ l volume of microspheres was added to tubes to give an initial ratio of 50 beads/cell. Fresh culture medium then was added to each tube to give a final volume of 2.0 ml/tube. This latter step prevents the development of acidic media conditions resulting from metabolic activity of the cells during incubation. Cells then were incubated overnight (18 hr) at 37°C and 5% CO₂. Following incubation, cells were washed twice with standard buffer to remove non-phagocytized microspheres, after which cells were resuspended in 0.5 ml standard buffer and immediately analyzed by flow cytometry. Before analysis the cytometer was standardized for fluorescence using a sample of cells without beads. Thus, the peak on the fluorescence histogram resulting from cells containing single microspheres could be identified. For each sample 10,000 events were collected and the number of cells ingesting fluorescent particles was expressed as a percentage of 10,000.

2.6. Plaque-forming cell (PFC) assay

Ability to produce specific antibody was determined by quantifying the plaque-forming cell (PFC) response to the T-dependent antigen, sheep red blood cells (SRBC) (Roitt and Delves, 1992). Mice were immunized by ip injection of 2×10^8 SRBC and IgM PFCs were enumerated in splenic cells 4 days later. Briefly, the spleen was collected under sterile conditions and immediately placed into 5 ml cold HBSS in sterile plastic 7 cm culture dishes (Fisher Scientific, Norcross, GA, USA). Splenic cells were separated by gentle maceration over

60 μ m autoclavable wire-mesh screening, transferred to 15 ml conical tubes (Fisher), and washed once in cold HBSS at 400 g, 7 min, 4°C. The resulting cellular pellet was resuspended in 4 ml cold complete medium and placed on ice. A 50- μ l aliquot was removed and combined with 450 μ l 10% trypan blue for enumeration and viability determination using a hemacytometer at 40 \times .

Splenic cell suspensions (2×10^7 cells/ml) were prepared in sterile culture medium. An aliquot of each sample (50 μ l, 10^6 cells) was mixed with 20 μ l guinea pig complement (Sigma), 30 μ l of 30% SRBC, and 0.4 ml warmed agar (47°C; Sigma) in a 35 \times 10 mm culture dish (Fisher), then incubated in 37°C and 5% CO₂ for 4 hr. After incubation, the number of plaques was counted under a light microscope. Results were expressed as number of PFC per 10^6 cells.

2.7. Statistical analysis

Data were expressed as arithmetic mean \pm SEM. Analysis of variance (ANOVA) was used with Dunnett's *t*-test to establish significant differences among groups. Experiments were performed in triplicate. Results described as different in this paper indicate significantly different at $P < 0.05$.

Table 1
Body weight in C57Bl/6 mice topically exposed to permethrin

Mouse body weight (g, means \pm SEM)			
Permethrin exposure	Days after dosing termination		
	2 day	10 day	30 day
Exposure 1:			
Control	20.81 \pm 0.65	23.20 \pm 0.60	22.40 \pm 0.30
0.5 μ l/day	21.70 \pm 0.45	23.20 \pm 0.20	23.29 \pm 0.47
1.5 μ l/day	20.61 \pm 0.48	21.10 \pm 0.40*	22.29 \pm 0.48
5.0 μ l/day	20.34 \pm 0.43	22.90 \pm 0.40	22.70 \pm 0.35
Exposure 2:			
Control	19.26 \pm 0.43	21.59 \pm 0.57	22.40 \pm 0.67
0.5 μ l/day	19.02 \pm 0.56	21.60 \pm 0.42	22.60 \pm 0.50
1.5 μ l/day	19.23 \pm 0.64	21.15 \pm 0.54	22.80 \pm 0.40
5.0 μ l/day	20.79 \pm 0.60	21.33 \pm 0.57	21.90 \pm 0.78
Exposure 3:			
Control	20.98 \pm 0.60	21.46 \pm 0.38	23.14 \pm 0.34
0.5 μ l/day	21.09 \pm 0.22	20.67 \pm 0.57	23.31 \pm 0.15
1.5 μ l/day	21.13 \pm 0.52	21.96 \pm 0.59	21.86 \pm 0.60
5.0 μ l/day	22.36 \pm 0.50	21.94 \pm 0.52	21.48 \pm 0.31
Exposure 4:			
Control	20.78 \pm 0.51	21.58 \pm 0.38	21.07 \pm 0.44
0.5 μ l/day	20.84 \pm 0.49	20.60 \pm 0.28	21.14 \pm 0.81
1.5 μ l/day	21.23 \pm 0.51	21.27 \pm 0.21	22.10 \pm 0.45
5.0 μ l/day	20.95 \pm 0.59	21.09 \pm 0.55	21.79 \pm 0.52

Exposure 1 = every day for 10 consecutive days.

Exposure 2 = every other day for 7 exposures.

Exposure 3 = every other day for 14 exposures.

Exposure 4 = every day for 30 consecutive days.

*Statistically significant ($P < 0.05$).

3. Results

3.1. Mouse body weights

Topical permethrin treatment did not affect body weight of experimental mice (Table 1). In one experiment (1.5 $\mu\text{l/day}$, with evaluation at 10 days post exposure) body weight of experimental mice was lower than control mice. No decrease or trend towards a decrease was present in the higher dose group in this particular experiment, or in the other 11 experiments, thus this single observation was felt to be due to chance rather than a result of chemical exposure.

3.2. Chemiluminescent response

Splenic macrophage number was determined by flow cytometry, and was not decreased in permethrin-exposed mice (unpublished data). A dose-related decrease in H_2O_2 production by splenic macrophages

was present in all exposure groups 2 days after termination of treatment (Fig. 1). This decrease remained significant 10 days after termination of permethrin treatment in exposures 1 and 4; trends towards decreased H_2O_2 production was present in exposure 3 10 days after termination of treatment, and in exposures 3 and 4 30 days after termination of treatment. Mean H_2O_2 production in exposure 2 mice (the group receiving the lowest total permethrin exposure) was increased at 10 and 30 days, a possible rebound effect.

3.3. Phagocytosis of fluorescent microspheres

A single significant inhibition of phagocytic ability of macrophages occurred 2 days after dosing ended, in the low dose group of exposure 4 (Table 2). Given the lack of a dose-response trend in this particular experiment, and lack of significant effect in other day 2 data, this single observation may not be biologically meaningful. Trends towards reduced phagocytosis were present in

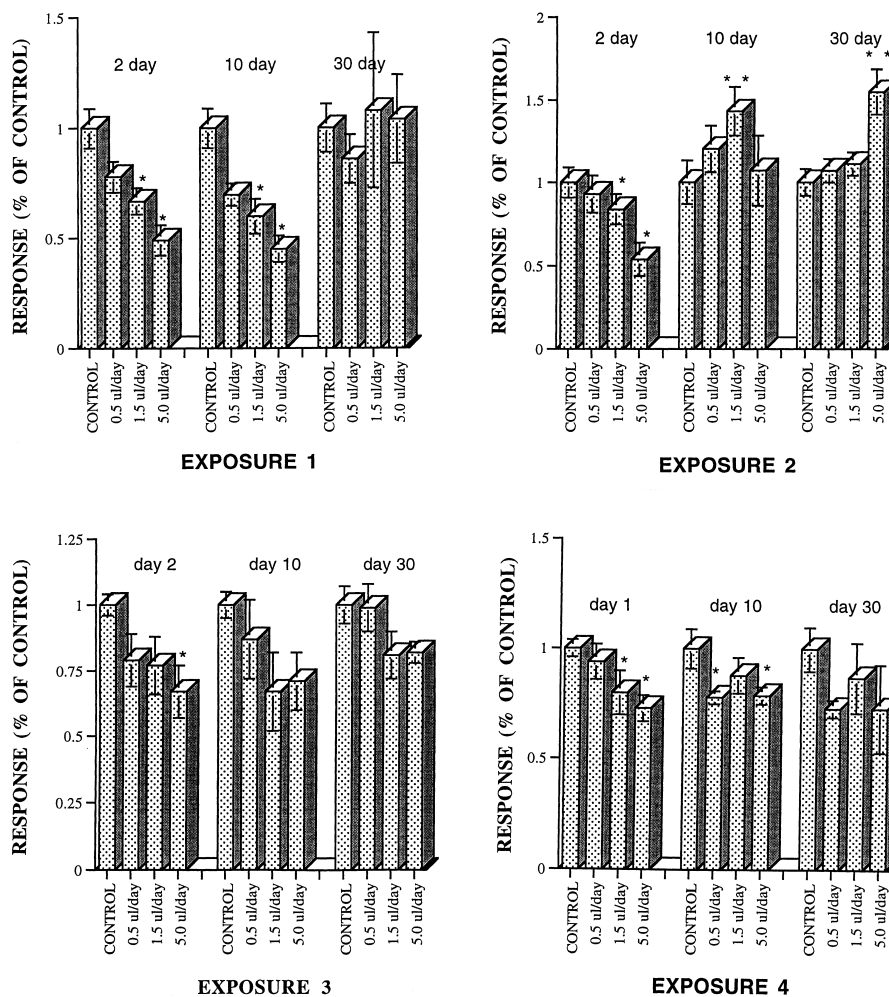


Fig. 1. Chemiluminescent response in splenic macrophages from mice exposed to permethrin by the topical route. Mice were evaluated 2, 10 or 30 days after termination of dosing. Topical exposure was to vehicle or 0.5, 1.5 or 5.0 μl permethrin for 10 or 30 days (exposures 1 and 4, respectively) or every other day for a total of 7 or 14 treatments (exposures 2 and 3, respectively).

Table 2
Macrophage phagocytosis in C57Bl/6 mice topically exposed to permethrin

Macrophage phagocytic activity			
Permethrin exposure	Days after dosing termination		
	2 day	10 day	30 day
Exposure 1:			
Control	1.00±0.10	1.00±0.11	1.00±0.14
0.5 µl/day	0.94±0.06	1.14±0.22	1.21±0.13
1.5 µl/day	1.13±0.12	0.98±0.12	1.23±0.09
5.0 µl/day	1.07±0.05	1.26±0.16	1.52±0.19
Exposure 2:			
Control	1.00±0.09	1.00±0.14	1.00±0.20
0.5 µl/day	0.82±0.07	1.03±0.25	1.56±0.31
1.5 µl/day	0.86±0.13	0.86±0.13	3.22±0.27**
5.0 µl/day	0.80±0.11	1.80±0.02**	2.20±0.30
Exposure 3:			
Control	1.00±0.53	1.00±0.69	1.00±0.23
0.5 µl/day	0.44±0.17	0.46±0.28	0.79±0.25
1.5 µl/day	0.34±0.11	0.30±0.26	0.82±0.14
5.0 µl/day	0.30±0.07	0.94±0.45	0.81±0.12
Exposure 4:			
Control	1.00±0.16	1.00±0.14	1.00±0.14
0.5 µl/day	0.62±0.10*	1.00±0.17	1.89±0.14
1.5 µl/day	1.02±0.04	0.80±0.13	1.67±0.00
5.0 µl/day	0.71±0.15	0.72±0.22	1.61±0.16

Exposure 1 = every day for 10 consecutive days.

Exposure 2 = every other day for 7 exposures.

Exposure 3 = every other day for 14 exposures.

Exposure 4 = every day for 30 consecutive days.

Data represent decimal portion of Control, and are presented as means±SEM.

*Significant decrease; **significant increase ($P < 0.05$).

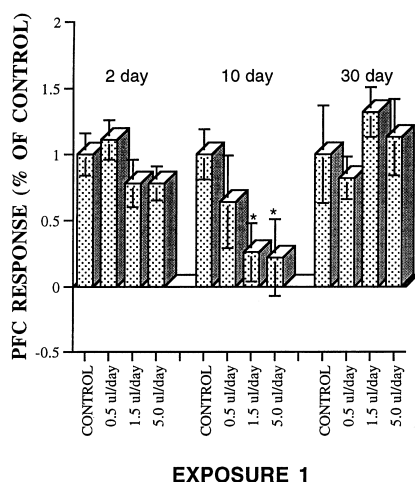


Fig. 2. Plaque-forming cell assay in splenic B lymphocytes from mice exposed to permethrin by the topical route. Mice were evaluated 2, 10 or 30 days after termination of dosing. Topical exposure was to vehicle or 0.5, 1.5 or 5.0 µl permethrin/day for 10 days.

exposures 2 and 3 mice. Similar to the chemiluminescent response, exposure 2 mice sometimes showed significant increased activity (phagocytosis) at days 10 and 30. Trends towards increased phagocytosis were also seen in exposure 1 and 4 mice at day 30.

3.4. PFC response

Permethrin produced a non-significant trend towards decreased plaque number in exposure 1 mice at 2 days after dosing termination (Fig. 2). This trend became significant in exposure 1 mice at 10 days post-dosing. Antibody production was not significantly affected by the other permethrin exposure regimens, nor were trends towards decreased or increased antibody production suggested in these groups (data not shown).

4. Discussion

Recent reports have demonstrated immune effects in experimental animals treated with pyrethroid insecticides. However, none of these reports examined the possibility that topical exposure (the most relevant route of human exposure) may be sufficient to alter function of the immune system. Percutaneous absorption of varying levels of permethrin has been demonstrated in the mouse (Shah et al., 1981), rhesus monkey (Sidon et al., 1988), rat, rabbit, dog and human (Taplin et al., 1990). Collectively, these reports raise questions about adverse immune effects in animals exposed to permethrin by the dermal route.

An effect of topical permethrin on humoral immune function was suggested in this study. Low-level subacute permethrin exposure inhibited the PFC response in mice treated daily for 10 days, but not in mice receiving intermittent or 30-day exposure. In what may be a related observation, we recently demonstrated thymic atrophy in mice exposed to 1.5–5.0 µl topical permethrin daily for 10 days, but not in mice exposed to the same doses every other day for 14 or 28 days, or daily for 30 days (Punareewattana et al., 2000). The reason for this difference in effect with different dosing schedules remains unclear. However, permethrin is rapidly metabolized by ester hydrolysis and oxidation (Casida et al., 1983) resulting in short half-life (12.37 hr in rats; Anadon et al., 1991). Thus, intermittent low-level exposure may be less likely to produce detectable systemic immune effects. It is also possible that hepatic enzyme-inducing ability of permethrin (Anadon et al., 1988) may lead to diminution of effect with 30-day exposure.

Regarding the ability of permethrin to depress antibody production, this has not been previously reported. However permethrin has been found to inhibit production of cytokines essential for antibody production (e.g. IFN γ and IL-4) (Diel et al., 1998) and also inhibited B

lymphocyte proliferation (Stelzer and Gordon, 1984). Further, oral cypermethrin (a cyano-substituted permethrin) decreased antibody production in both mice and rats (Desi et al., 1985; Tamang et al., 1988).

An effect of topical permethrin on the ability of splenic macrophages to produce H_2O_2 was clearly evident in the present study. The inhibition of this response at 2 days post-exposure was consistent across all dose groups, dose-dependent, and persisted up to 10 days after dosing termination. Mechanisms leading to this effect are not yet known; however, Gassner et al. (1997) demonstrated that permethrin inhibits mitochondrial complex I, which may interfere with the electron transport chain and thus the respiratory metabolic burst of phagocytic cells.

The observation of inhibited immune responses in rodents treated with topical permethrin raises questions about risk to humans exposed by the topical route to this common insecticide. The spray application of military clothing with 0.5% permethrin resulted in a predicted absorbed dose of 0.14 $\mu\text{g}/\text{kg}/\text{day}$ by the wearer of such clothing (Snodgrass, 1992). Shah et al. (1981) reported that skin penetration of permethrin in mice measured 88% of the applied dose within 8 hr of treatment. Using this figure, the present low-dose mice treated with 22 mg/kg/day permethrin would absorb about 19 mg/kg/day. This is about 135,000 times higher than the 0.14 $\mu\text{g}/\text{kg}/\text{day}$ predicted for humans wearing permethrin-treated clothing.

Use of permethrin-containing products as pediculicides and scabicides may result in considerably higher levels of human permethrin exposure, especially in children (Vander Stichele et al., 1995). Systemic absorption of about 124 $\mu\text{g}/\text{kg}$ permethrin would be predicted in a 22.5 kg child using NIX[®] (1% permethrin) for treatment of head lice (CEPA, 1992). Assuming the 88% absorption rate reported by Shah et al. (1981) in mice, the present low-dose mice received about 158 times this dose daily for 10 or 30 days, or every other day for 14 or 28 days.

Studies using topical Elimite[®] creme (5% permethrin) in humans have shown that about 1.25% of the permethrin is absorbed after an 8–14 hr treatment (i.e. after treatment according to label for mites) (COT, 1994). Elimite[®] is described as safe and effective in children of 2 months of age and older [Physicians Desk Reference (PDR), 1995]. Based on this estimated 1.25% absorbed rate, an 11.2-kg child treated with 10 g Elimite[®] (containing 500 mg permethrin) would receive about 6.25 mg absorbed dose, corresponding to approximately 558 $\mu\text{g}/\text{kg}$. Again assuming an 88% absorption rate in mice, the present low dose mice received 34 times this dose per day.

No data are available for percutaneous absorption of permethrin from corn oil (the present vehicle). Further, although Shah et al. (1981) observed 88% absorption of

permethrin in acetone within 8 hr of treatment in mice, the same laboratory, using a nearly identical protocol, later reported mouse skin penetration of 2.5% in 8 hr and 26% in 48 hr (Grissom et al., 1987). Sidon et al. (1988) reported percutaneous absorptions of permethrin in propylene glycol of 22%, 9% and 44% for pesticide applied to monkey forehead, monkey forearm, and rat back, respectively. In *in vitro* studies, Baynes et al. (1997) reported 1.2–1.7% permethrin in DMSO or acetone crossed the skin of mice.

The upper end of estimated permethrin transfer across mouse skin in acetone (88%) used for comparative purposes in the above discussion is 70.4 times the estimated transfer rate across human skin (1.25%) from a crème vehicle. It is unknown whether this may overestimate permethrin absorption from an oil-based (corn oil) vehicle. The latter possibility should be considered, in that should the low-end reported permethrin transfer rate across mouse skin (2.5%; Grissom et al., 1987) more closely predict absorption from corn oil, then systemic exposure in the present low-dose mice would be estimated at 550 $\mu\text{g}/\text{kg}/\text{day}$, a level nearly identical to the 558 $\mu\text{g}/\text{kg}$ reported in humans using Elimite[®]. Accurately determining percutaneous transfer of permethrin across mouse skin from corn oil will therefore be an important next step for estimating risk to human immune health from topical application of permethrin.

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