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HYDROCARBON-BASED WEAPONS MAINTENANCE COMPOUNDS PRODUCE EVIDENCE OF CONTACT HYPERSENSITIVITY IN BALB/C MICE

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Unprotected dermal contact with weapons maintenance materials is highly probable during cleaning and maintenance of firearms. Several weapons maintenance materials of interest to the Department of Defense were evaluated for their irritating and sensitizing potential in a modified local lymph node assay (LLNA). Female BALB/c mice (n = 5) were topically exposed to Break-Free CLP, Royco 634, TW-25B, MC-25, or MC-2500. All compounds tested produced a positive response for irritancy and lymphocyte proliferation. Break-Free CLP and Royco 634 produced the greatest dermal irritation and highest LLNA stimulation index. Phenotyping of draining lymph node cells from animals treated with Break-Free CLP suggest that this material induces T-cell-mediated contact sensitization (Type IV hypersensitivity) in mice. These findings support the recommendation that persons handling or using weapons maintenance materials should protect their skin from repeated contact by wearing appropriate personal protective equipment.

Keywords: Contact hypersensitivity; Dermal irritation; Hydrocarbons; Organic esters; Polyalphaolefin

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Animal Use statement: The experiments reported here were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, DHHS, Publication No (NIH) 86–23 (1996). Procedures involving mice were approved by the NIOSH (Morgantown, WV) Institutional Animal Care and Use Committee (IACUC). These studies were supported in part by NIEHS intra-agency agreement # Y1-ES-0001-03.

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INTRODUCTION

Cleaning and maintenance of firearms and large caliber weapons is an integral task for many military personnel, police, firearm enthusiasts, and hunters. The potential for dermal exposure to weapons maintenance materials is significant given the hands-on nature of weapons cleaning and the infrequent use of personal protective equipment (PPE) during these activities. There are several products available specifically designed for the cleaning and maintenance of firearms that have been considered for use by the U.S. Department of Defense (US-DOD). Break-Free CLP, manufactured by Armor Holdings, Inc., has been the standard weapons maintenance material of the U.S. Armed Forces since 1979 and has also been adopted by military and security services of a number of countries including Austria, Belgium, Canada, Holland, Italy, Norway, Sweden, and Germany. Royco 634, manufactured by Royal Lubricants, Inc., is a competing product of Break-Free CLP also in use with the US-DOD. Both materials are similar in composition in that they contain hydrocarbon and synthetic oils but each contains different additives. MC-2500 and TW-25B, manufactured by Mil-Comm Products, Inc., are marketed as replacement weapons maintenance materials for Break-Free CLP/Royco 634 and differ somewhat in composition from the latter in that they contain synthetic oils/highly refined mineral oils. MC-25, also manufactured by Mil-Comm Products, Inc., is marketed as an environmentally safe, water-based cleaner/degreaser for use in cleaning firearms. The chemical composition of MC-25 is not available.

Chronic, unprotected exposure to these materials is a concern in that some weapons maintenance materials have been identified as dermal irritants in mice (1–3). Recent studies found that repeat dermal application of Break-Free CLP to the backs of CD-1 mice resulted in the accumulation of transient erythematous dermal irritation characterized histopathologically by markedly thickened epithelium and inflammatory infiltrates over 10 days in five of 20 mice (2,3). Continued application of Break-Free CLP 3 times a week for 90 days resulted in dermal irritation in a majority (15 of 20) of mice (1). Also, *in vitro* dermal penetration studies showed that Break-Free CLP penetrated into and accumulated in mouse, rat, and pig skin supporting the hypothesis that Break-Free CLP has the potential to induce contact sensitization through interacting with Langerhans cells situated within the suprabasel layer of the epidermis (4).

Given the potential widespread use of weapons maintenance materials and the previous findings in mice, studies were undertaken to evaluate several materials of interest to the Department of Defense (Break-Free CLP, Royco 634, MC-2500, TW-25B, MC-25) for their potential to induce dermal irritancy and sensitization.

METHODS AND MATERIALS

Chemicals

Break-Free CLP liquid was purchased from Break-Free Inc., Armor Holdings (Jacksonville, FL). Royco 634 was purchased from Royal Lubricants, Inc. (East Hanover, NJ). MC-2500, MC-25, and TW-25B were purchased from Mil-Comm Products, Inc. (Wallington, NJ). With the exception of Break-Free, all weapons maintenance products were diluted in 99% acetone. Break-Free was diluted in

99% acetone: olive oil (1:1). Dilutions of 25% and 50% and neat material of the weapons maintenance products were used in the described tests.

Alpha-hexylcinnamaldhyde (HCA, 85% pure) and toluene 2,4-diisocyanate (TDI, 99.6% pure) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). The HCA (30% in 99% acetone) was used as the positive control for the LLNA and TDI (2.5% in 99% acetone) was used as the positive control for phenotypic analysis assay.

All positive control and test dilutions were prepared daily and used within 30 minutes of preparation.

Animals

Female BALB/c mice, 6–8 weeks of age, were purchased from Taconic Laboratories (Germantown, NY) or Charles River Laboratories (Wilmington, MA). Mice were housed under NIH animal care guidelines in the AAALAC-accredited NIOSH animal facility (Morgantown, WV). A 12-hour light/dark cycle was maintained and target temperature and humidity ranges were 18–26°C and 30–70%, respectively. Animals were weighed, individually identified by tail mark and assigned to homogenous weight groups. They were housed five per group and Agway Prolab 3500 diet and tap water were available *ad libitum*. Mice were allowed to acclimate for a minimum of 5 days prior to the onset of study. Animals were weighed immediately before sacrifice in all studies to access chemical-induced toxicity.

Local Lymph Node Assay (LLNA)

The LLNA was performed following the protocol recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Immunotoxicology Working Group (5) with minor modifications. Mice were topically exposed to 25 μL of vehicle, test article, or positive control on the dorsal surface of each ear daily for 3 consecutive days. For the study comparing the five neat materials a naïve control group was included. On day 6, all mice were administered 0.2 mL (20 μCi) ^{3}H-thymidine (Perkin Elmer, MA) by intravenous tail vein injection. Five hours after injection, mice were euthanized via CO_2 overdose and a single-cell suspension was prepared from pooled right and left draining lymph nodes (DLN) for each animal. The uptake of ^{3}H-thymidine was determined using a β-scintillation counter, and a stimulation index (SI) was determined for each experimental group by dividing the group mean by the mean of the vehicle controls. A chemical was classified as positive if at least one concentration reached a stimulation index (SI) of ≥ 3 with the results being statistically significant and dose-responsive.

Evaluation of Irritancy

Prior to the first exposure ear thickness measurements were taken for both left and right ears of each animal. Measurements were taken using a modified Mitutoyo micrometer (Mitutoyo, Japan) and averaged to establish a baseline ear thickness. Animals were then exposed to neat test article for 3 consecutive days as described

earlier for the LLNA. One group of animals remained naïve and served as the control group. Twenty-four hours after the final exposure, measurements were taken and percent ear swelling was calculated using the following formula:

$$\% \text{ ear swelling} = \frac{(\text{posttreatment measure} - \text{pretreatment measure})}{\text{pretreatment measure}} \times 100 \qquad (1)$$

HISTOPATHOLOGY

A separate group of animals that had not been exposed to ^{3}H-thymidine were used for histopathological evaluation (n = 2 for all groups except TW-25B where n = 5). Animals were exposed as described previously for the LLNA and ear thickness measurements were taken prior to the first exposure and 24 hours following the final exposure. On day 6, the study animals were euthanized and the ears were excised and preserved in 10 mL of 10% phosphate-buffered formalin and processed using standard techniques. The tissues were embedded in paraffin, cut to 5 μm, and stained with hematoxylin and eosin.

Phenotypic Analysis

Mice (n = 5) were exposed dermally to 25 μL neat Breakfree CLP, acetone (the vehicle for TDI), or 2.5% TDI as described earlier for the LLNA, for 4 consecutive days. One group remained naïve. Additional groups were added to this study in which the skin was breached using a Multii-Test II® device (Lincoln Diagnostics, Inc., Decatur, IL) prior to each exposure. Mice were rested for 6 days and then sacrificed on day 10. Cardiac puncture to collect sera was performed following CO_2 euthanasia, and right and left DLNs from each individual mouse were removed and placed in 2 ml PBS. Single cell suspensions were made and phenotypic analysis of DLN cells was conducted as described previously, gating on viable nonred blood cells (6). Cells were stained using monoclonal antibodies against IgE (FITC; clone R-35–72) and B220 (PE; clone RA3-6132). The Fc block used was anti-mouse CD32/16 (clone 2.4 G2). All antibodies were purchased from PharMingen (San Diego, CA). Results were expressed as a percentage of gated cells and as absolute numbers. Sera were used for analysis of total serum IgE by ELISA as described later.

Total Serum IgE Analysis

Total serum IgE was quantified following a standardized ELISA procedure as described by Manetz and Meade (6) with minor modifications. Plates were coated with 0.2 μg rat α-mouse IgE monoclonal Ab (B1E3). The B1E3 hybridomas were generously provided by Daniel Conrad (Virginia Commonwealth University, Richmond, VA). Test sera were applied to the plates at an initial dilution of 1:40 and serially diluted (1:2) down eight wells. A purified mouse IgE clone IgE-3 anti-trinitrophenyl (anti-TNP, PharMingen, Torreyanna, CA) was used as the standard, at a starting concentration of 5000 ng/mL and serially diluted (1:2) through 12 wells. The secondary antibody, biotinylated antimouse IgE, clone R35–92 (PharMingen), was used at a concentration of 0.2 μg. A 1:400 dilution of streptavidin alkaline

phosphatase (SAP, Sigma-Aldrich) was used, followed by detection with substrate [*p*-nitrophenyl phosphate tablets (Sigma-Aldrich) added to substrate buffer]. Plates were read on a Beckman Vmax model plate reader at wavelengths of 405 and 650 nm, and data were analyzed with Softmax 3.1.1 ELISA software (Molecular Devices, Sonnyvale, CA). Plates were analyzed when the standard reached an OD of at least 1.5, but did not exceed 2.1. Test samples were quantitated by comparison to the standard curve.

Statistical Analysis

One way analysis of variance (ANOVA) was performed to analyze variability between at least three experimental groups. If a p value of 0.05 was achieved, Dunnett's post test was performed to compare test groups to their appropriate control. When only two groups were analyzed, a *t*-test was performed. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Initial studies were conducted to evaluate the sensitization potential of the weapons cleaning compounds using the LLNA. All five compounds induced dose responsive lymph node cell proliferation with the SI of the neat materials ranging from 6.7 for MC-25 to 13.7 for Royco 634 (Fig. 1). The positive control (30% HCA in acetone) for the assays was within the historical control range for this lab in all studies with SI ranging from 10.7 to 10.9. All animals exposed to the weapons cleaning compounds exhibited gross signs of irritation with mild to severe hyperemia

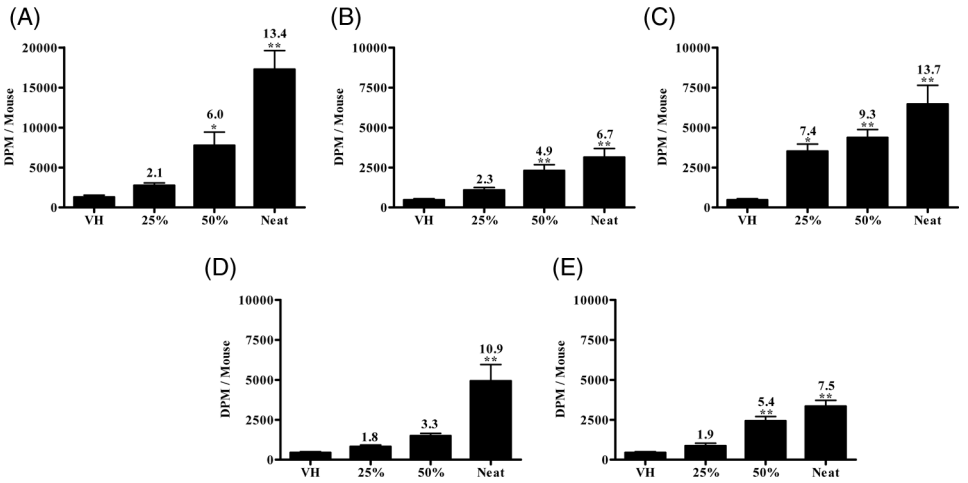

Figure 1 Analysis of the sensitization potential of Break-Free CLP (A), MC-25 (B), Royco 634 (C), MC-2500 (D), or TW-25B (E) using the LLNA. ^{3}H-thymidine incorporation into DLN cells of BALB/c mice following exposure to vehicle or the concentration of weapons cleaning material shown. Data are expressed as group mean DPM (n = 5) ± standard error. Numbers appearing above the bars represent the stimulation indices for each concentration tested. $^{*}p < 0.05$ as compared to VH control and $^{**}p < 0.01$ as compared to VH control using a Dunnett's test.

of the ears. Animals exposed to Break-Free CLP and Royco 634 exhibiting the most severe reactions including alopecia of the skin between the ears. There were no significant changes in the average body weights of exposed animals suggesting that exposure did not induce severe systemic toxicity. Daily observations did not identify any clinical signs or behaviors suggesting that toxicity occured in exposed animals. However, pathological evaluations of the major tissues of these animals were not performed. Therefore, it is not certain if exposure resulted in toxicity independent of body weight change or clinical behavior suggestive of toxicity.

In order to better compare the sensitization potentials of the weapons cleaning compounds and evaluate the irritancy response, the neat materials of all five compounds were tested simultaneously in a single combined irritancy/LLNA assay. A naïve group was included as a negative control. As in the previous studies, all compounds yielded a positive response with SI for Break-Free CLP > Royco 634 > MC-2500 ~ TW-25B ~ MC-25 (Fig. 2). Consistent with the previously observed hyperemia and alopecia, Break-Free CLP induced the highest degree of ear swelling with over a 100% increase in ear thickness. Although Royco 634 induced alopecia in animals, the percentage increase in ear thickness in these animals was 52%, comparable to that induced by MC-2500, which induced minimal alopecia (Fig. 2).

Histopathological evaluation of the ears of mice exposed to each of the weapons cleaning compounds was consistent with previously observed ear swelling data. All exposed animals exhibited some degree of epithelial hyperplasia with hyperkeratosis and dermal inflammatory cell (primarily neutrophilic) infiltration. The reaction was most severe in the animals exposed to Break-Free CLP (Fig. 3B) and the Royco 634 (Fig. 3D).

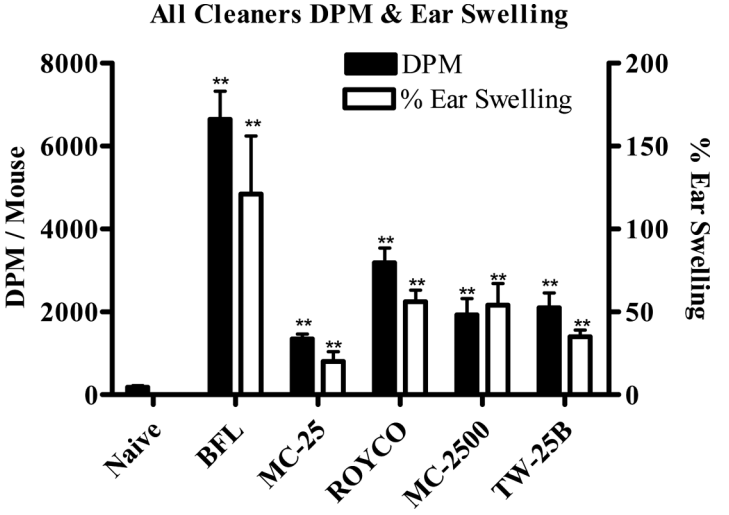

Figure 2 Percent ear swelling following gun cleaner materials exposure at 24 hours post challenge and the analysis of sensitization potential by the LLNA. Light bars represent mean % ear swelling (n = 7 except TW-25B n = 5) ± standard error and the dark bars represent DPM/mouse (n = 5) ± standard error. **indicates a significant difference at $p < 0.01$ when compared to the Naive control group using the Student's *T*-test.

Table 1 Analysis of the phenotype of draining lymph node cells and average total serum lgE levels from animals topically exposed to neat Break-Free CLP

	Phenotyping					
	B220+		IgE+B220+		Total Serum IgE (ng/ml)	
Group	Intact	Abraded	Intact	Abraded	Intact	Abraded
Naive	19.6 ± 1.2 (4.0 ± 0.7)	22.1 ± 1.7 (6.0 ± 0.9)	0.9 ± 0.4 (0.1 ± 0.02)	1.9 ± 0.9 (0.1 ± 0.1)	289 ± 48.8	398 ± 66.7
Break Free	$29.1 \pm 1.2^{**}$ (6.4 ± 0.4)	$30.7 \pm 1.0^{**}$ (7.6 ± 0.7)**	$8.3 \pm 1.9^{**}$ (1.6 ± 0.4)**	$7.6 \pm 1.3^{**}$ (1.7 ± 0.4)**	366 ± 53.6	547 ± 116.0
Acetone	20.7 ± 0.7 (1.3 ± 0.04)		0.5 ± 0.2 (0.04 ± 0.02)		222 ± 26.0	
TDI (2.5%)	$43.5 \pm 1.2^{**}$ (19.3 ± 2.2)**		$20.2 \pm 2.1^{**}$ (6.2 ± 1.0)**		$1536 \pm 314.7^{*}$	

Data represent the mean percent of the indicated cell phenotype or total serum IgE concentration per group ($n = 5$). The numbers in parentheses represent the absolute cell concentration ($\times 10^6$) for each group. *indicates $p < 0.05$, **indicates $p < 0.01$ by Student *T*-test.

As the main focus of this work was the evaluation of the irritancy and sensitization potential of Break-Free CLP, an additional study was conducted with this compound to evaluate its potential to induce IgE production (Table 1). Phenotypic analysis of draining lymph node cells from animals topically exposed to neat Break-Free CLP demonstrated a significant increase over control of both the percentage and absolute numbers of $B220^+$ cells (29.1% vs.19.6% and 6.4×10^6 vs. 4.0×10^6, respectively). Although statistically significant, the increase in IgE^+B220^+ cells (8.3% vs. 0.9%) was not considered to be biologically significant and was within the historical control range for this laboratory. Additionally, breaching of the skin prior to chemical exposure did not alter the response (Table 1). Serum IgE levels of mice exposed to Break-Free CLP were not significantly elevated over controls in animals that had exposure to either intact (366 ng/mL vs. 289 ng/mL) or abraded (547 ng/mL vs. 398 ng/mL) skin. Animals exposed to the positive control, 2.5% TDI, demonstrated elevated levels compared to controls of $B220^+$ cells (43.5% vs. 20.7%), IgE^+B220^+ cells (20.2% vs. 0.5%, respectively), and serum IgE levels (1536 ng/mL vs. 222 ng/mL, respectively).

DISCUSSION

The weapons maintenance materials evaluated in these studies are currently in use or being considered for use by the Department of Defense. Both Break-Free CLP and Royco 634 are combination weapons maintenance materials in that they are designed for cleaning, lubricating, and preserving weapons. Both contain hydrocarbons and synthetic oils, but differ in that Break-Free CLP contains a significant amount of dibasic ester #1 whereas Royco 634 contains calcium dinonylnaphthalenesulfonate and methyl oxirane. TW-25B is a lubricating grease that is specified for use in several large bore weapons systems by the U.S. Army, Navy, and Air Force and is used in the manufacture of a large range of bore weapons and military-style knives. MC-2500 oil is a pourable form of TW-25B. The main ingredients of both

materials are synthetic oils/highly refined mineral oils, a calcium sulfonate/carboxylate complex, and a phosphate ester (7,8). MC-25 is described as a nontoxic, water-based cleaner/degreaser for metal parts. The chemical composition of MC-25 is not available.

The results of these studies suggest that all of the weapons maintenance materials tested have potential to induce dermal irritation and contact sensitization. Of the materials tested, Break-Free CLP induced the most robust response in both the LLNA and ear swelling assay and, correspondingly, the most severe skin reactions in terms of epithelial hyperplasia and dermal inflammatory response. Repeat application of Break-Free CLP to the ears of female BALB/c mice resulted in a dose-dependent increase in lymphocyte proliferation and an increase in the $B220^+$ cell population without a corresponding increase in serum IgE and IgE^+B220^+ cells, suggesting that the response was T-cell rather than IgE-mediated (6). These findings are consistent with previous studies conducted in our laboratory that showed that repeat topical application of Break-Free CLP to the backs of CD-1 mice over 90 days resulted in erythematous dermal irritation characterized by thickened epithelium and inflammatory infiltrates at the application site (1–3). Dermal penetration studies showed that Break-Free CLP accumulates in skin suggesting that the material would be available for interaction with Langerhan's cells and the induction of contact sensitization (4).

The MSDS for Break-Free CLP indicates that the material contains polyalphaolefin oil (65%), synthetic oils, esters, and synthetic proprietary ingredients (27%); isoparaffinic hydrocarbons (5%); and dibasic ester (3%) (9). Several of these ingredients have been identified as dermal irritants. Isoparaffinic hydrocarbons and synthetic oils (10–13) as well as dibasic ester #1 (14) have been shown to induce dermal irritation in laboratory animals and humans. Polyalphaolefin oil has been shown to induce dermal irritation (15). Undecane has been identified as a major component of Break-Free CLP by GC-MS (4) and is a potent skin irritant (16). As reviewed by ATSDR (15), 2 U.S. military polyalphaolefin hydraulic fluids, MJL-H83232LT and B85–174, displayed skin sensitization activity in guinea pigs (17,18) suggesting that this material could be the ingredient in Break-Free CLP that promotes skin sensitization in mice. Isoparaffinic hydrocarbons are not known to induce contact sensitization (11,19). No information was found on the sensitizing potential of dibasic ester #1.

Royco 634 induced lower levels of lymphocyte proliferation and was less irritating to mouse skin producing relatively less alopecia at the application site as compared with Break-Free CLP. Royco 634 and Break-Free CLP both contain petroleum distillates. However, unlike Break-Free CLP, Royco 634 does not contain isoparaffinic hydrocarbons or dibasic ester #1. Petroleum distillates are not known to induce sensitization reactions (20) and studies have found that isoparaffinic hydrocarbons rarely induce dermal irritation in nonoccluded tests (11). In order to identify the irritating substances in Break-Free CLP, future testing should focus on the contribution of isoparaffinic hydrocarbons, dibasic ester #1, and the other components of Break-Free CLP, individually and in combination.

TW-25B is a grease and MC-2500 is the "pourable" form of TW-25B. According to their MSDS, MC-2500 and TW-25B are similar in chemical composition explaining the similar results for these materials in the LLNA/irritancy assay. The

chemical composition of MC-25 has not been reported by the manufacturer. This material is a cleaner/degreaser and it is expected that its chemical composition would differ from the lubricating compounds, TW-25B and MC-2500.

The results of these studies suggest that repeat contact with Break-Free CLP and the other weapons cleaning materials tested could result in dermal irritation and/or contact sensitization. Based on these findings and others (1–3), persons handling or using weapons maintenance materials should wear polyethylene, Neoprene, or PVC gloves to protect their skin from exposure and the potential development of allergic and irritant contact dermatitis. Wearing protective gloves may not always be practical (e.g., military field environment), but persons should be aware that repeat or chronic dermal contact with weapons maintenance materials should be avoided if possible. Exposed skin should be decontaminated by washing with soap and water when practical, as indicated in the manufacturers MSDS.

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