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RESEARCH ARTICLE

Evaluation of irritancy and sensitization potential of metalworking fluid mixtures and components

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Abstract

There are approximately 1.2 million workers exposed to metalworking fluids (MWF), which are used to reduce the heat and friction associated with industrial machining and grinding operations. Irritancy and sensitization potential of 9 National Toxicology Program (NTP) nominated MWFs (TRIM 229, TRIM VX, TRIM SC210, CIMTECH 310, CIMPERIAL 1070, CIMSTAR 3800, SYNTILO 1023, SUPEREDGE 6768, and CLEAREDGE 6584) were examined in a combined local lymph node assay (LLNA). BALB/c mice were dermally exposed to each MWF at concentrations up to 50%. Significant irritation was observed after dermal exposure to all MWFs except CIMTECH 310 and SYNTILO 1023. Of the 9 MWFs, 6 induced greater than a 3-fold increase in lymphocyte proliferation and 7 tested positive in the irritancy assay. TRIM VX yielded the lowest EC3 value (6.9%) with respect to lymphocyte proliferation. Chemical components of TRIM VX identified using HPLC were screened for sensitization potential using structural activity relationship (SAR) modeling and the LLNA. TOPKAT predicted triethanolamine (TEA) as a sensitizer while Derek for Windows predicted only 4-chloro-3-methylphenol (CMP) to be positive for sensitization. When tested in the LLNA only CMP (EC3 = 11.6%) and oleic acid (OA) (EC3 = 29.7%) were identified as sensitizers. Exposure to all tested TRIM VX components resulted in statistically significant irritation. An additive proliferative response was observed when mixtures of the two identified sensitizing TRIM VX components, OA and CMP, were tested in the LLNA. This is one explanation of why the EC3 value of TRIM VX, with respect to lymphocyte proliferation, is lower than those assigned to its sensitizing components.

Keywords: *metalworking fluids; LLNA; contact dermatitis; irritancy*

Introduction

Approximately 1.2 million workers are potentially exposed to metalworking fluids (MWF) in machine finishing, machine tooling, and other metalworking and metal-forming operations (NIOSH, 1998a). MWF are used to reduce heat and friction associated with industrial machining and grinding operations and to ultimately improve product quality. There are numerous types of MWF ranging from straight oils to water-based fluids including soluble, semi-synthetic, and synthetic fluids (Verma et al., 2006). MWF are often complex mixtures of oils, emulsifiers, anti-weld agents, buffers, biocides, and other additives. The fluid complexity is compounded by contamination with substances from industrial use that encourage microbial growth, which can introduce biological contaminants such as endotoxins, exotoxins, and mycotoxins (MMWR, 2002; Sloyer et al., 2002; Li et al., 2003).

MWF exposure can occur through inhalation of the aerosols generated in the machining process or through skin contact when parts, tools, and equipment covered with the fluids are handled. Dermatologic exposures

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are most commonly associated with, but not limited to, allergic and irritant dermatitis (Tapp, 2007). Contact dermatitis of the hands and forearms in workers exposed to MWF is a widespread problem (de Boer et al., 1989a, 1989b). Studies have shown the prevalence of dermatitis to be between 20–30% among workers who handle MWF, much higher than the 4% recorded among the general population (Sprince et al., 1996). The causes of dermatitis in these workers are likely to be multi-factorial and include exposure to a wide variety of metal types, different types of MWF solvents, biocidal additives, and exposure to damaged skin. The prognosis for MWF dermatitis may be poor, as shown by a study of 100 machine operators with documented MWF dermatitis (Pryce et al., 1989a). In a 2-year follow-up, 78% of those who continued working with soluble oils had not healed; 70% of those who discontinued contact with the MWF had not healed (Pryce et al., 1989a and b).

Occupational exposures to MWF have been identified to cause a variety of other health effects including respiratory conditions such as hypersensitivity pneumonitis (HP), chronic bronchitis, impaired lung function, and asthma (Glaser et al., 2003). There is also evidence that chronic exposures to some MWF are associated with increased risk of certain types of cancer, such as breast and prostate (Agalliu et al., 2005; Thompson et al., 2005). Little information is provided by manufacturers about the chemical makeup of specific MWF due to industry competition and trade secrets; however, analytical techniques can be used to separate, identify, and study MWF constituents.

The purpose of this study was to use a combined irritancy/local lymph node assay (LLNA) to investigate the sensitization and irritancy potential of the nine MWF nominated by the National Toxicology Program (NTP) for carcinogenicity studies. To identify the potential allergen(s) in TRIM VX, the MWF identified to induce the most robust proliferative response, constituents were identified using HPLC analysis, their sensitization potential was predicted using structure activity relationship (SAR) modeling and the irritancy and sensitization potential of each constituent was evaluated using a combined LLNA. In an attempt to explain the mechanism behind the lymphocyte proliferation induced by exposure to TRIM VX, mixtures of the sensitizing components were also examined in the LLNA.

Materials and methods

Animals

Female BALB/c mice, 8–12-wk-old, were purchased from Taconic (Hudson, NY). Mice were quarantined in the NIOSH Animal Facility for 1 wk upon arrival and maintained under conditions specified by NIH guidelines. Animals were fed a modified NIH-31 6% irradiated rodent diet (Harlan Teklad #7913) and provided tap water *ad libitum*. Animal facilities were maintained between 18–26°C and 25–70% relative humidity with light–dark cycles at 12-hr intervals (6:00–18:00). Cages were cleaned and sanitized weekly. The NIOSH Animal Facility is an environmentally controlled barrier facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Mice were weighed, tail-marked for identification, and assigned to homogeneous weight groups ($n=5$) before each experiment.

Test articles

TRIM® VX, TRIM® 229, TRIM® SC210, CIMSTAR® 3800, CIMPERIAL® 1070, CIMTECH® 310, SYNTILO 1023, SUPEREDGE 6768, and CLEAREDGE 6584 were provided by the National Toxicology Program (NTP). Triethanolamine (TEA; CAS# 102-71-6), 4-chloro-3-methylphenol (CMP; CAS# 59-50-7), α-terpineol-(p-menth-1-8-ol) (TPM; CAS# 10482-56-1), oleic acid (OA; CAS# 112-80-1), α-hexylcinnamic aldehyde (HCA; CAS# 101-86-0) and dinitro-fluorobenzene (DNFB; CAS# 885-62-1) were purchased from Sigma Aldrich (St Louis, MO).

Range finding and toxicological studies

Range finding studies were performed to select the concentrations of MWF or component to be used for irritancy/sensitization studies. For these studies, mice (three per group) were dosed with vehicle (50% ethanol or 4:1 acetone/olive oil) or concentrations of the test article (up to 50% based on limits of solubility) on the dorsal surface of each ear (25 µl per ear) for 3 consecutive days. The highest soluble concentrations were selected for these studies in an attempt to identify toxicity. Animals were allowed to rest for 2 d following the last exposure and then were weighed and examined for signs of toxicity including change in body weight, ruffled fur or fluid from orifices. At the end of the study, mice were euthanized by CO_2 inhalation. For the subsequent studies, maximum concentrations were selected that were soluble in the vehicle and did not cause toxicity (NIEHS, 1999). Ethanol (50%) was used as the vehicle for TRIM® VX, TRIM® 229, TRIM® SC210, CIMSTAR® 3800, CIMPERIAL® 1070, CIMTECH® 310, SYNTILO 1023, SUPEREDGE 6768, and CLEAREDGE 6584. A 4:1 dilution of acetone/olive oil was used as the vehicle for TEA, CMP, TPM and OA.

Irritancy

Evaluation of irritancy was performed as previously described (Woolhiser et al., 1998). Briefly, before the first MWF administration, the thickness of the right and left pinnae of each mouse ear was measured using a modified engineer's micrometer (Mitutoyo Co., Kanagawa, Japan). Mice were exposed to 25 µl of vehicle or test article for 3 consecutive days. Ear thickness measurements

were taken 24 hr following the final exposure. The mean percentage of ear swelling was calculated based on the following equation: [(mean post-challenge ear thickness – mean pre-challenge ear thickness)/mean pre-challenge thickness] × 100. DNFB was included as a positive control in these studies. MWF and TRIM VX components were tested at concentrations up to 50%. To reduce the number of animals required, these evaluations were conducted in conjunction with the LLNA described next.

Local lymph node assay of MWF

The LLNA was performed following the method described in the ICCVAM Peer Review Panel report (NIEHS, 1999) with minor modifications as previously described by Anderson et al. (2007). BALB/c mice were used for these studies and have been shown to have a comparable LLNA response to the recommended CBA strain (Azadi et al., 2004). Mice (5 per group) were exposed topically with designated vehicle, increasing concentrations of test article(s), or positive control (30% HCA) on the dorsal surface of each ear (25 µl per ear) for 3 consecutive days. MWF and TRIM VX components were tested at concentrations ranging from 6.25%–50%. The LLNA has not been accepted by regulatory agencies for investigations into the sensitization potential of mixtures. It was used in these studies to compare the dose-response curve for lymphocyte proliferation. A dose-responsive, statistically significant increase in lymphocyte proliferation yielding an Stimulation Index (SI) value above 3 is the criterion for the identification of positive LLNA response in these studies. EC3 values (concentration required to induce a 3-fold increase over the vehicle control) were calculated based on the equation as described previously (Basketter et al., 1999, 2003).

Chemical analysis of TRIM VX

The reagents used for the analysis of TRIM VX include: water, carbon disulfide, hexane, 2-propanol, acetonitrile, and methanol obtained as the highest purity available from Burdick & Jackson (Honeywell Corp., Morristown, NJ). Formic acid, pro analysis grade (Acros Chemicals, Morris Plains, NJ) was added to water at 0.1% (v/v) to make HPLC mobile phase A. Mobile phase B was made from 33.3% 2-propanol and 66.6% acetonitrile, and mobile phase C was made from 33.3% 2-propanol and 66.6% hexane. A "universal solvent mixture" (USM) was prepared from methanol, 2-propanol, and hexane (1:1:1 v/v/v) and used to make the MWF concentrate samples at 0.05 g/ml concentrations. The analytical HPLC method added the internal standards pyridine, caffeine, bisphenol-A, terphenyl, and octadecylbenzene (Sigma) to TRIM VX at concentrations of 25 ng/ml, 87.9 µg/ml, 1871 µg/ml, 92 µg/ml, and 3962 µg/ml, respectively. The GC-MS method used the ASTM D2887-01 Calibration Mix (Catalog #31674, Restek, Bellefonte, PA) that is a 1% w/w mixture of n-alkanes C5-C44 in carbon disulfide diluted either 1:100 or 1:1000 as an internal or external standard.

Analytical HPLC analysis

The HPLC system consisted of an HP-1050 quaternary solvent pumping system, automated sample injector, column heater, and UV detector (Agilent Technologies, Palo Alto, CA). UV light absorbance was monitored at 254 nm for analyte detection. An evaporative light scattering detector (ELSD) was placed after the UV detector (Model Sedex 55, S.E.D.E.R.E., France). The analytical column was a 3.0 mm × 250 mm XTerra® MS-C18 column with 5 µm particles (Waters, Milford, MA). Mobile phase flow rate was 0.5 ml/min at 40°C and the injection volume was 10 µl. This method used a ternary mobile phase system, three mobile phases A, B, and C. The mobile phase gradient blending program started with 100% mobile phase A blended to 100% mobile phase B in 60 minutes, and then to 100% mobile phase C in the next 30 min for a 90-min run time. It was critical that the mobile phase system returned to mobile phase A in reverse sequence using short 10-min steps. Detector response data was acquired using a Dionex ADC interface unit and the data was analyzed using Dionex AI-450 Chromatography Data Acquisition Software run on a Microsoft Windows operating system and Dell PC (Dionex Corp., Sunnyvale, CA).

Semi-preparative HPLC analysis

A semi-preparative HPLC system was used to isolate specific components of TRIM VX. The semi-preparative HPLC system was the same as the analytical system, but with a larger column, faster flow rates, and larger injection volumes without the use of the destructive ELSD. The mobile phase flow was diverted to collection vessels while the components of interest eluted. The semi-preparative column was a 10 mm × 300 mm XTerra® MS-C18 column (Waters) that had a 10 µm particle size, a 0.65 cm^3/g pore volume, and an average pore diameter of 113 Å. Mobile phase flow rate was 5.0 ml/min at 40°C, and the injection volume was 100 µl. Ten 100-µl injections into the system before the mobile phase gradient program started allowed for maximum loading of the column, 75 mg. After fractionation, the isolates were subjected to other analytical techniques as needed like gas chromatography-mass spectroscopy (GC-MS). This technique allowed for the purification and concentration of specific components in a metalworking fluid. The HPLC solvent fractions were injected directly into the GC-MS with a proper solvent delay.

GC-MS analysis

TRIM VX and fractionated components were also analyzed by direct injection cool on column gas chromatography

with mass spectrometry detection (COC-GC-MS). The GC-MS system consisted of an Agilent 6890N GC, a 5973 inert MSD with both electron impact (EI) and chemical ionization (CI) ion sources, a 7683 autosampler, and a 7683 injection tower. The chemical ionization mode used methane gas. The samples were directly injected onto a 530 µm ID 5 meter long deactivated fused silica retention gap and separated on a 250 µm ID 30 meter long HP-5si capillary column. The carrier gas was helium at a flow rate of 1.0 ml/min. The MSD transfer line temperature was 325°C. The oven program had an initial temperature of 30°C that upon injection was ramped at 5.32°C/min to 350°C in a 60 min run time. Samples were injected with a 0.1 µl volume and analyzed using electron impact, positive chemical ionization, and negative chemical ionization modes. The mass spectrometer scanned a mass range from 2 to 800 amu, and used a quad temperature of 150°C, a source temperature of 230°C, and an electron multiplier voltage of about 1294 volts. CMP and TEA in TRIM VX were confirmed by matching spectrum and retention times to certified external standards, and the other components were identified only by matching spectrum with the NIST library spectrum to a 95% quality level as defined by the Agilent software.

Structural activity relationship modeling

Two commercial software packages, TOPKAT 6.2 (Accelrys, Inc., San Diego, CA) and Derek for Windows version 9.0.0 (Lhasa Limited, Leeds, UK) were used to estimate the skin sensitization potential of the TRIM VX components tested in the LLNA.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 5.0 (San Diego, CA). All data were analyzed by a one-way analysis of variance (ANOVA) and when significant differences were detected ($p = 0.05$), Dunnett's test was used to compare treatment groups with the appropriate control group. If the assumptions were not able to be met by parametric analysis, the nonparametric Kruskal–Wallis k-sample test was utilized followed by the Mann–Whitney U test for pairwise comparisons with the control. Statistical significance is designated by $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$.

Results

Evaluation of toxicity

All MWF and TRIM VX components were evaluated for toxicity using a range finding study. At the concentrations tested, no overt toxicity or body weight loss was observed following exposure to any of the test articles (data not shown).

Irritancy

The results of the irritancy studies for the nine MWF are presented in Table 1. Significant ear swelling was observed after exposure to the maximum concentration of 50% for TRIM VX, TRIM 229, SUPEREDGE 6768, TRIM SC210, CIMPERIAL 1070, CLEAREDGE 6584 and CIMSTAR 3800. No significant irritation was observed after exposure to CIMTECH 310 or SYNTILO 1023 at concentrations of 50%. The percent ear swelling identified for the vehicle control fell within the historical range. In all experiments, the positive control (DNFB) resulted in a significant ear swelling response ($p < 0.01$) when compared to the 50% ethanol exposed group.

Local lymph node assay for MWF

LLNA results for the nine MWF are presented in Table 1. The soluble MWF [SUPEREDGE 6768 (EC3 = 11.7%), TRIM VX (EC3 = 6.9%), and CIMPERIAL 1070 (EC3 = 29.9%)] were all identified to induce a 3-fold increase in lymphocyte proliferation in the LLNA, with TRIM VX yielding the lowest EC3 value, identifying it as a good candidate for additional studies. Significant lymphocyte proliferation was observed after exposure to concentrations of 25% TRIM VX and SUPEREDGE 6768 and 50% CIMPERIAL 1070. Statistically significant proliferation in the draining lymph nodes was observed after exposure to the semi-synthetic MWF CLEAREDGE 6584 (25%) and CIMSTAR 3800 (50%) with EC3 values of 8.7% and 31.0%, respectively. Insignificant results were obtained for TRIM SC210 at all concentrations tested although an EC3 value of 48.1% was calculated. Of the synthetic MWF, only TRIM 229 (EC3 = 42.1%) tested positive in the LLNA with statistically significant proliferation after exposure to 50%. Negative results were obtained for CIMTECH 310 and SYNTILO 1023 at all concentrations tested. The DPM identified for the vehicle control fell within the historical range. In all experiments, the positive control (30% HCA) resulted in a significant response ($p < 0.01$) when compared to the vehicle (50% ethanol) group.

Identification of TRIM VX components

UV absorption and evaporative light scattering detection (ELSD) were used to determine TRIM VX components (Figure 1A). The identified components were: (1) triethanolamine, (2) 4-chloro-3-methylphenol, (3) hexadecanoic acids, (4, 5, 6, 7) octadecanoic acids, (8, 9, 10) the methyl esters of hexadecanoic acid acids and octadecanoic acids, (11, 12, 13, 14) chloroparaffins, and (15) mineral oil. The ELSD response is more proportional to mass than UV detector for compounds larger than C14. The filled peaks are internal standards. The semi-preparative fractionation chromatograms for TRIM VX are presented in Figure 1B.

Analyte detection was achieved by non-destructive UV absorbance and the internal standards served as benchmarks to mark collection times for components that were identified by ELSD. For example, fraction F9 contained chloroparaffin as confirmed by gas

Table 1. Irritancy and lymphocyte proliferation of MWFs.

Type of Metal Working Fluid	Metal Working Fluid	LLNA (DPM ± SE, SI)	Irritancy (% Ear Swelling)	LLNA EC3
SOLUBLE	Trim VX			
	0%	914 ± 120	0.5 ± 0.4	6.9
	10%	3483 ± 559, 3.8	16.2 ± 0.6**	
	25%	5327 ± 1070**, 5.8	19.4 ± 0.2**	
	50%	9597 ± 947**, 10.5	18.3 ± 0.1**	
SOLUBLE	SUPEREDGE 6768			
	0%	732 ± 127	0.8 ± 1.4	11.7
	10%	1941 ± 300, 2.7	9.6 ± 2.4	
	25%	4478 ± 571**, 6.1	23.2 ± 3.9**	
	50%	5946 ± 565**, 8.1	25.5 ± 3.8**	
SOLUBLE	CIMPERIAL 1070			
	0%	1301 ± 239	2.9 ± 2.3	29.9
	10%	1520 ± 362, 1.2	4.5 ± 2.8	
	25%	2570 ± 373, 2.0	16.6 ± 4.5	
	50%	9747 ± 938**, 7.5	29.1 ± 2.4**	
SEMI-SYNTHETIC	CLEAREDGE 6584			
	0%	805 ± 173	−0.1 ± 2.3	8.7
	10%	2794 ± 362, 3.5	4.0 ± 2.0	
	25%	5332 ± 738**, 6.6	23.3 ± 2.8**	
	50%	6314 ± 734**, 7.8	37.2 ± 5.7**	
SEMI-SYNTHETIC	TRIM SC210			
	0%	1067 ± 223	0.9 ± 2.9	48.1
	10%	2667 ± 728, 2.5	7.4 ± 3.1	
	25%	3039 ± 685, 2.8	15.9 ± 2.0**	
	50%	3485 ± 667, 3.3	31.3 ± 3.6**	
SEMI-SYNTHETIC	CIMSTAR 3800			
	0%	1301 ± 239	2.9 ± 2.3	31.0
	10%	1789 ± 214, 1.4	2.8 ± 2.7	
	25%	3172 ± 570, 2.4	7.2 ± 2.9	
	50%	6495 ± 916**, 5.0	17.9 ± 2.2**	
SYNTHETIC	TRIM 229			
	0%	914 ± 120	0.5 ± 0.4	42.1
	10%	1145 ± 77, 1.3	4.3 ± 0.8**	
	25%	1361 ± 248, 1.5	5.3 ± 0.7**	
	50%	3338 ± 600**, 3.7	12.5 ± 0.5**	
SYNTHETIC	SYNTILO 1023			
	0%	732 ± 127	0.8 ± 1.4	–
	10%	723 ± 142	−0.6 ± 2.8	
	25%	1062 ± 209, 1.5	8.2 ± 1.3	
	50%	1165 ± 177, 0.6	5.8 ± 2.1	
SYNTHETIC	CIMTECH 310			
	0%	1067 ± 223	0.9 ± 2.9	–
	10%	929 ± 129	−3.0 ± 2.6	
	25%	811 ± 144	7.5 ± 0.6	
	50%	975 ± 50	−2.1 ± 1.4	

Values represent the mean ± SE derived from 5 animals/group. [SI = Stimulation Index]

* Significantly different from 50% ethanol vehicle controls, $p \leq 0.05$.

** Significantly different from 50% ethanol vehicle controls, $p \leq 0.01$.

EC3 values represent an estimate for comparison purposes because the samples are compound mixtures.

chromatography-atomic emission detection (GC-AED) analysis for carbon, hydrogen, and chlorine. HPLC fractionation resolved components that would be masked by interferents using direct GC-MS. Figure 2 is an example of TRIM VX analysis by GC-MS using these three ionization modes, electron impact ionization, positive chemical ionization and negative chemical ionization. Analysis of TRIM VX components using classical electron impact

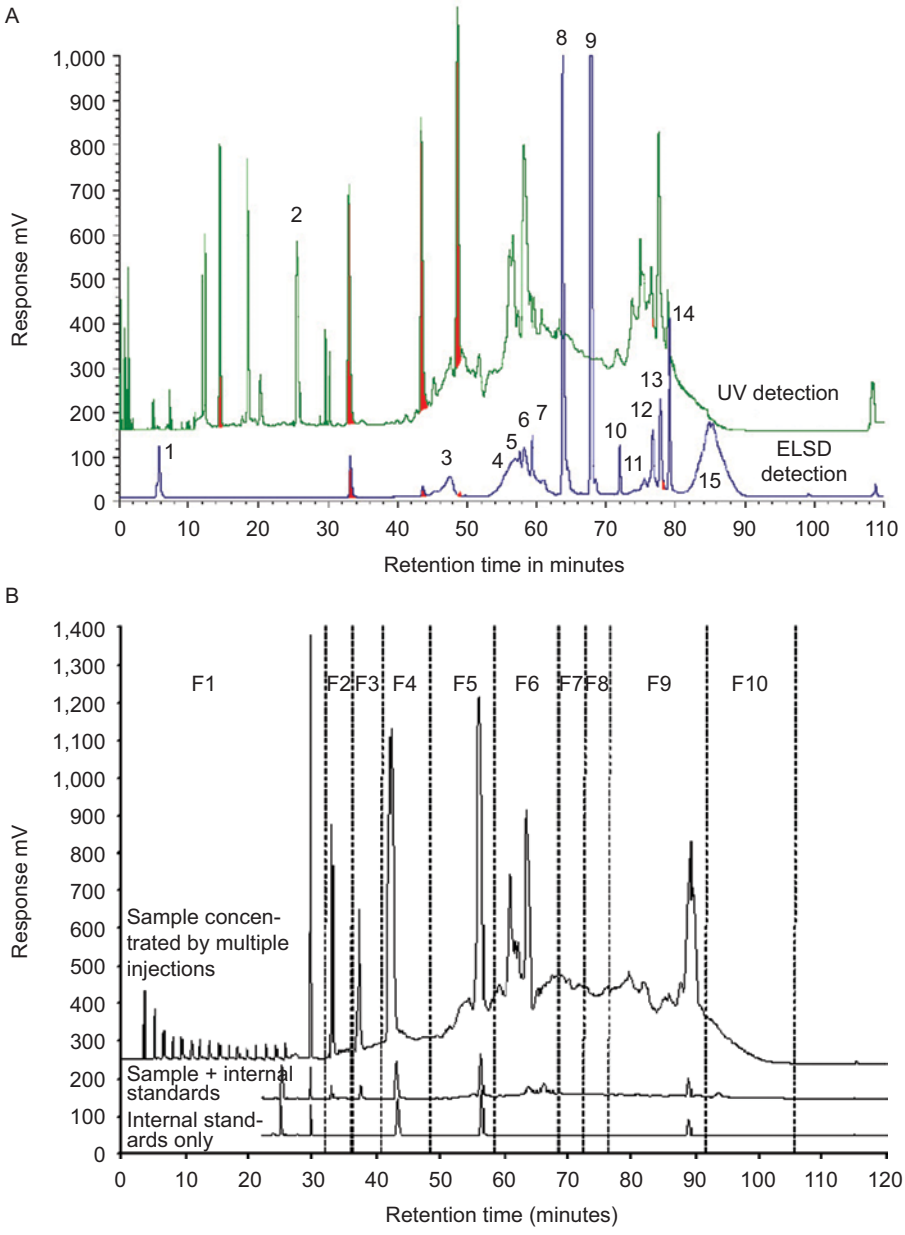

Figure 1. (A) The HPLC-UV-ELSD analysis of Trim VX. Analysis of TRIM VX using ELSD detection (lower chromatogram) UV adsorption detection (upper chromatogram). The major components were (1) triethanolamine, (2) 4-chloro-3-methylphenol, (3) hexadecanoic acids, (4, 5, 6, 7) octadecanoic acids, (8, 9, 10) the methyl esters of hexadecanoic acids and octadecanoic acids, (11, 12, 13, 14) chloroparaffins, and (15) mineral oil. (B) Semi-preparative fractionation chromatograms of TRIM VX. Presented in this chromatogram are internal standards (bottom chromatogram), metalworking fluid sample plus internal standards (middle chromatogram), and the sample concentrated by multiple injections and separated into 10 fractions, F1 to F10 (top chromatogram).

(EI) ionization mode (bottom chromatogram) provided a spectrum for library search, match, and identification. The major components identified by GC-EI-MS were (1) (+)-α-terpineol, (2) 4-chloro-3-methylphenol, tripropylene glycol, and tripropylene glycol methyl ether, (3) triethanolamine borate, (4) hexadecanoic acid methyl ester, (5) 8-octadecenoic acid methyl ester, (6) octadecanoic acid methyl ester, (7) 9-octadecenoic acid (oleic acid), (8) Bisphenol-A (IS), (9) dodecylbenzene (IS), (10) chloroparaffins, and (11) mineral oil and other minor components. Positive chemical ionization (middle chromatogram) identified amines like triethanolamine borate and provided molecular ions for molecular weight determination. Negative chemical ionization (top chromatogram) was selective for chloroparaffins and provided molecular ions for molecular weight determination.

SAR modeling of TRIM VX components

The commercially-available TRIM VX components identified by chemical analysis were evaluated for

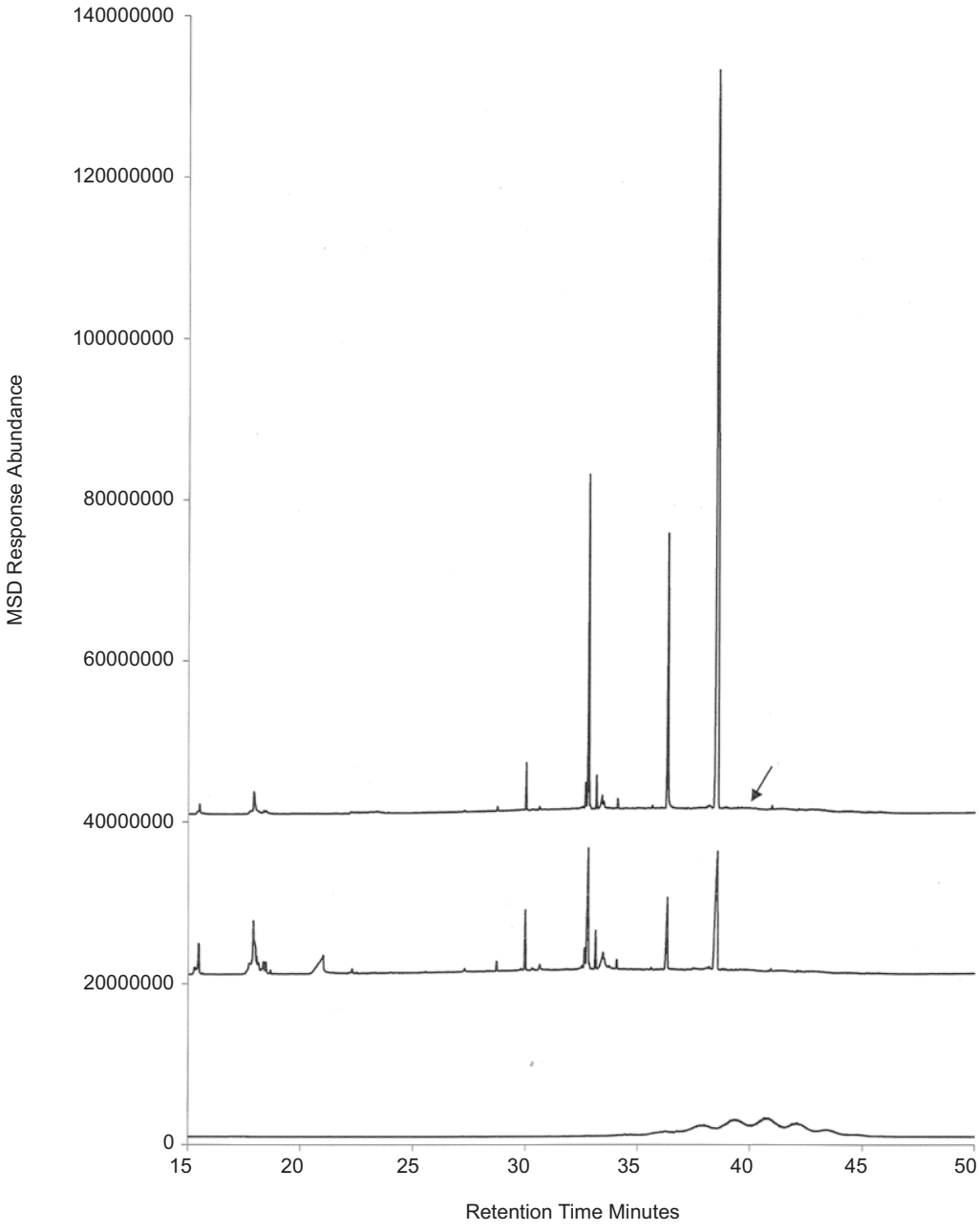

Figure 2. TRIM VX analysis using GC-MS. Analysis of TRIM VX components using classical electron impact (EI) ionization mode is presented in the top chromatogram, positive chemical ionization in the middle chromatogram, and negative chemical ionization in the bottom chromatogram. The major components are identified by number: (1)(+)-α-terpineol, (2) 4-chloro-3-methylphenol, tripropylene glycol, and tripropylene glycol methyl ether, (3) triethanolamine borate, (4) hexadecanoic acid methyl ester, (5) 8-octadecenioc acid methyl ester, (6) octadecanoic acid methyl ester (7) 9-octadecenoic acid (oleic acid) (8) Bisphenol-A (IS) (9) dodecylbenzene (IS), (10) chloroparaffins, and (11) mineral oil.

sensitization potential using two software packages, TOPKAT and Derek for Windows (Table 2). The potential for Triethanolamine (TEA) to induce a sensitization response was predicted by TOPKAT 6.2. The potential for 4-chloro-3-methylphenol (CMP) to induce sensitization was predicted by Derek for Windows but not by TOPKAT. Neither program predicted the methyl esters, oleic acid (OA), or α-terpineol-(*p*-menth-1-8-ol) (TPM) as potential sensitizers.

Local lymph node assay and irritancy responses of TRIM VX components

SAR modeling has not been accepted as a replacement for validated animal models for analyzing sensitization potential; therefore TRIM VX components were evaluated for sensitization potential using the LLNA. Commercially available components of TRIM VX were evaluated for irritancy and sensitization potential using the combined LLNA (Table 3). All evaluated components

Table 2. Identification and SAR evaluation of skin sensitization activity of TRIM VX constituents.

Name	TOPKAT		DEREK for Windows
	Activity	Severity	
Triethanolamine	+	Weak	—
4-chloro-3-methylphenol	—	Inactive	+
(+)-α-terpineol (p-menth-1-8-ol)	—	Inactive	—
Eicosatrienoic acid methyl ester	—	Inactive	—
Octadecatrienoic acid methyl ester	—	Inactive	—
Octadecadienoic acid methyl ester	—	Inactive	—
Octadecenoic acid methyl ester	—	Inactive	—
Oleic acid	—	Inactive	—
Hexadecanoic acid methyl ester	—	Inactive	—

Table 3. LLNA/Irritancy dose-response studies for TRIM VX components.

TRIM VX Component	LLNA (DPM ± SE, SI)	Irritancy (% Ear Swelling)	LLNA EC3
Triethanolamine (TEA)			
0%	797 ± 104	2.8 ± 1.0	—
10%	977 ± 141, 1.2	–1.7 ± 3.1	
25%	1469 ± 211, 1.8	24.0 ± 3.9**	
50%	1884 ± 275, 2.4	24.2 ± 2.7**	
4-chloro-3-methylphenol (CMP)			
0%	797 ± 104	2.8 ± 1.0	11.6
5%	1977 ± 385, 2.5	7.5 ± 3.5	
10%	2032 ± 498, 2.5	18.9 ± 6.1	
25%	5349 ± 816**, 6.7	38.8 ± 6.2*	
α-terpineol-(p-menth-1-8-ol) (TPM)			
0%	543 ± 50	4.2 ± 2.0	—
10%	627 ± 48, 1.2	–2.5 ± 2.4	
25%	835 ± 60, 1.5	5.9 ± 0.8	
50%	1213 ± 60, 2.2	25.0 ± 3.3**	
Oleic Acid (OA)			
0%	1634 ± 226	–1.8 ± 2.0	29.7
10%	2629 ± 141, 1.6	11.2 ± 2.6	
25%	3884 ± 293, 2.4	29.2 ± 3.9	
50%	9077 ± 1557**, 5.6	51.6 ± 6.2**	

Values represent the mean ± SE derived from 5 animals/group. [SI = Stimulation Index]
* Significantly different from acetone/olive oil vehicle controls, $p \leq 0.05$.
** Significantly different from acetone/olive oil vehicle controls, $p \leq 0.01$.

of TRIM VX were identified as irritants (Table 3) when animals were dosed with the maximum concentration of 50%. In all experiments, the positive control (DNFB) resulted in a significant response ($p < 0.01$) when compared to the acetone/olive oil exposed group (data not shown). TEA and TPM were not identified as sensitizers in the LLNA, while exposure to CMP (EC3 = 11.6%) and OA (EC3 = 29.7%) resulted in significant sensitization responses at concentrations of 25% and 50%.

Local lymph node assay response of OA and CMP mixtures

In an attempt to explain the low EC3 value obtained for TRIM VX compared to the EC3 values for CMP and OA, mixtures of the two sensitizing components were examined in the LLNA. Shown in Figure 3A are the average SI values obtained from three individual LLNA experiments for CMP and OA. Exposure to low (6.25%) and high (25%) concentrations of OA were examined in combination with concentrations of CMP ranging from 6.25–25%. The average SI value for mixtures of 6.25% OA and ranging concentrations of CMP are presented in the solid line box above the bars in Figure 3B. The average SI value for mixtures of 25% OA and ranging concentrations of CMP are presented in the solid line boxes above the bars in Figure 3C. The animals in the 25% CMP/25% OA group had to be removed from the study due to toxicity. The predicted SI values, based on the addition of the individual SI values for CMP and OA (Figure 3A), are presented in the dashed line boxes above the bars (Figure 3B and 3C).

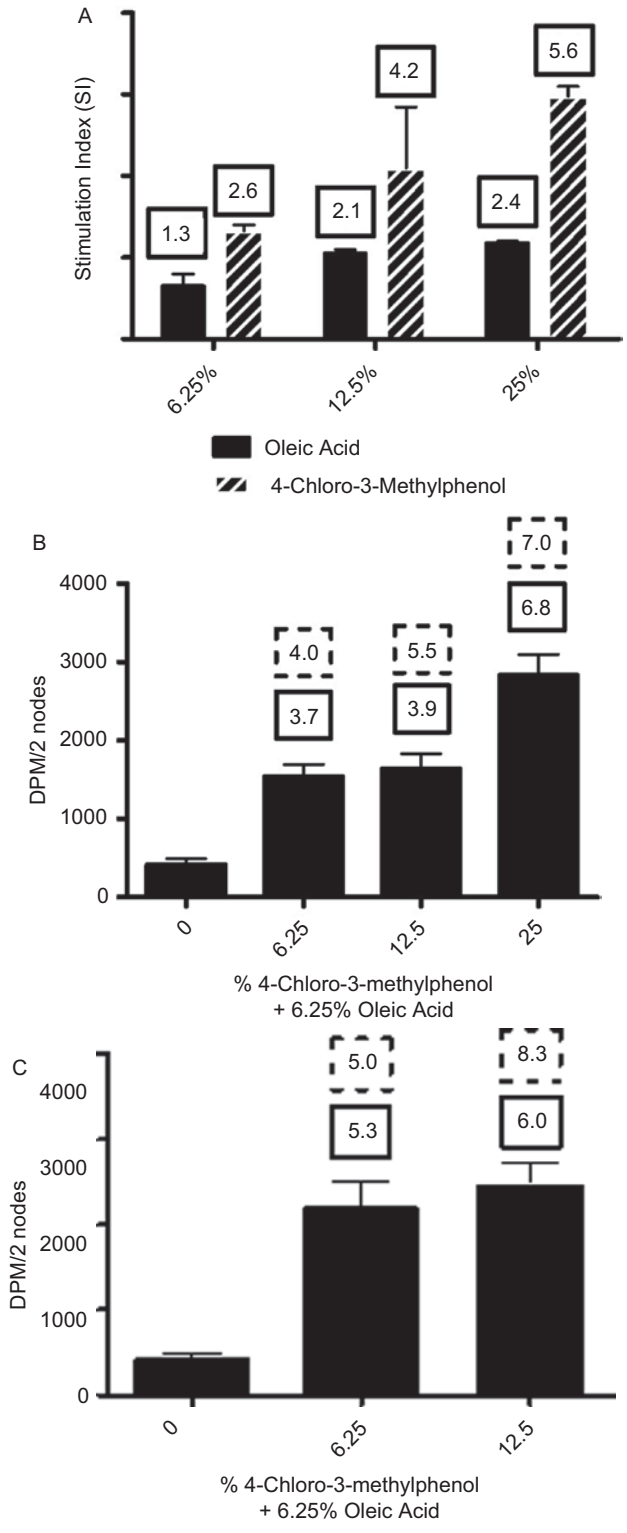

Figure 3. LLNA mixture studies for CMP and OA. Dose response curves for the lymphocyte proliferative response after exposure to CMP or OA (A) and mixtures of CMP and OA (B and C). The average SI value obtained from three LLNA experiments is reported for all studies. The numbers above the bars in the solid line box represent the actual SI value while the numbers in the dashed line box represent the predicted SI values.

Discussion

In this study, a combined irritancy/LLNA was used to screen compound MWF mixtures for potential adverse immune effects. These studies demonstrate the use of a tiered approach using the HPLC, GC-MS, SAR modeling, and LLNA to identify sensitizing components of MWF. The described method may be helpful in screening potentially sensitizing mixtures and identifying the responsible component(s) while excluding non-sensitizing mixtures. The LLNA has not been accepted by regulatory agencies for investigations of the sensitization potential of mixtures and the calculation of an EC3 value was formulated to determine the potency of individual chemicals, not mixtures. For these studies the EC3 value was used to compare the dose response lymphocyte proliferation curves of the MWF as a plausible screening tool to identify mixtures worthy of additional studies.

All of the soluble MWF examined in this study tested positive for irritancy and induced a 3-fold increase in lymphocyte proliferation. TRIM VX yielded the lowest EC3 value (6.9%) and was identified to be an irritant at concentrations as low as 10%. For purposes of hazard identification, the MWF tested in these studies were examined at concentrations higher than the manufacturer's recommended concentration (10%) for use. However, TRIM VX induced a positive response in mice after dermal exposure at concentrations lower than the manufacturer's recommended concentration and thus was targeted for component analysis. CLEAREDGE 6584 (EC3 = 8.7%) and SUPEREDGE 6768 (EC3 = 11.7%) induced positive responses after dermal exposure in mice at concentrations close to those recommended for use by the manufacturer.

TRIM VX is a soluble oil or chemical emulsion coolant concentrate designed for use with the heaviest duty machine operations. Soluble MWF are used to cool and lubricate in order to prevent welding of the cutting tool to the work surface, reduce abrasive wear of the tool at high temperatures, and prevent distortion caused by residual heat. Soluble MWF concentrates are diluted with water before use. They contain surface-active emulsifying agents to maintain the oil-water mix as an emulsion (Cookson, 1971; Menter et al., 1975). Super-fatty emulsions of soluble MWF are produced by the addition of fatty oil, fatty acids, or esters (NIOSH, 1998b).

The specific constituents of TRIM VX were identified using HPLC and GC-MS analysis and tested in the LLNA in an attempt to further define potential sensitizing agent(s). SAR modeling was used in conjunction with the LLNA in an attempt to decrease the number of animals needed for these types of studies. However, SAR modeling did not contribute to the hazard identification of these chemicals as results were not found to be consistent between SAR programs or between SAR and the LLNA.

In these studies, CMP and OA were identified as the most potent sensitizers with EC3 values of 11.6% and 29.7%, respectively. Previous research has found TRIM VX to contain about 2% of CMP and 10–20% of oleic acid methyl esters (Brown, 2008). The available data on the safety assessment of dermal exposure of CMP in MWF is insufficient. CMP is a biocide used in products such as cosmetics, industrial adhesives, industrial coatings, emulsions, paints and metal cutting fluids to control slime-forming bacteria and fungi that might develop. This chemical is not considered by the EPA to be a sensitizer (USEPA, 1997). Studies using human patch testing of the general public found 2% of 1462 subjects to be allergic to CMP (Andersen, 2006). The results presented here identify CMP as both an irritant and sensitizer when tested in a murine model.

OA is considered less hazardous than most chemicals and is a high volume chemical with production exceeding 1 million pounds annually in the United States (USEPA, 1990).

It is an unsaturated fatty acid that is often used as a corrosion inhibitor, a paint solvent, and can be used in pesticide products. Exposure data on this chemical is also lacking. Using a murine model, these studies have identified OA as both a contact sensitizer and irritant when tested in a murine model. OA is used as a lubricant in MWF and its content in some MWF can be >80%, which raises concern over exposure risk. TPM is a basic component of perfumes, cosmetics, and flavoring. Consistent with the results presented here TPM has been previously identified as a skin, eye, and respiratory irritant. TEA is also a high volume chemical and is suspected to be an immunotoxicant, respiratory toxicant, and skin or sense organ toxicant (USEPA, 1990). It is used in at least seven industries including the manufacturing of pesticide products. TEA is also suspected to be an animal carcinogen and may cause occupational asthma (Savonius et al., 1994). At the concentrations tested in these studies, this chemical was only identified as an irritant. TEA is typically used in MWF to stabilize pH or inhibit corrosion and can be used at concentrations up to 25%.

Additional investigations were conducted in an attempt to explain the low EC3 value obtained for TRIM VX compared to the EC3 value for the two identified sensitizing components, OA, and CMP. The predicted SI values (addition of the SI values for the individual chemicals) for the OA and CMP mixtures were similar to the calculated SI values (Figures 3B and 3C) following testing of the mixtures. This suggests the lymphocyte proliferative response of the CMP and OA mixture is the additive response of the two chemicals. However, this does not fully explain the low EC3 value for TRIM VX (6.9%) compared to its two identified sensitizing components, OA (29.7%) and CMP (11.6%).

Several mechanisms could be responsible for differences in the lymphocyte proliferation of mixtures compared to the individual sensitizing constituents including additive (as demonstrated in these studies), synergistic or antagonistic reactions between the chemical components. A synergistic effect could result from enhanced penetration of a particular chemical in a mixture, increased solubility in a vehicle or adjuvant activity of one chemical in the mixture increasing the allergenicity of a second chemical. In contrast, an antagonistic effect could result from anti-inflammatory properties of a compound in the mixture or decreased penetration or solubility in a vehicle.

There is also the possibility of chemical reactions occurring that result in the formation of new antigens. Skin patch testing has revealed that workers with allergic contact dermatitis may not have an allergic response to individual constituents of MWF, but they may instead respond to the reaction products of the MWF constituents. Shrank (1985) demonstrated negative patch test results when the 14 individual constituents of the MWF in question were tested. The specific allergen was only identified when constituents of each stage of the MWF manufacturing process were tested in combination.

In summary, seven of the nine NTP-nominated MWF tested positive in the irritancy assay. Six of the MWF tested yielded a 3-fold increase in lymphocyte proliferation in the LLNA. Three of these MWF induced a greater than 3-fold increase in lymphocyte proliferation at the recommended working concentrations raising concern over the potential of these mixtures to induce sensitization. HPLC and GC-MS analysis were used to identify the components of TRIM VX, the MWF identified to yield the largest increase in lymphocyte proliferation. When these components were tested in the LLNA, CMP and OA were identified as potential sensitizing components. These studies demonstrate the use of a tiered approach to identify sensitizing components of MWF.

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References

Agalliu, I., Eisen, E.A., Kriebel, D., Quinn, M.M., and Wegman, D.H. 2005. A biological approach to characterizing exposure to metalworking fluids and risk of prostate cancer (United States). *Cancer Causes Control* 16:323–331.

Andersen, A. 2006. Final report on the safety assessment of sodium *p*-chloro-*m*-cresol, *p*-chloro-*m*-cresol, chlorothymol, mixed cresols, *m*-cresol, *o*-cresol, *p*-cresol, isopropyl cresols, thymol, *o*-cymen-5 -ol, and carvacrol. *Int. J. Toxicol.* 25(S1):29–127.

Anderson, S.E., Wells, J.R., Fedorowicz, A., Butterworth, L.F., Meade, B.J., and Munson, A.E. 2007. Evaluation of the contact and respiratory sensitization potential of volatile organic compounds generated by simulated indoor air chemistry. *Toxicol. Sci.* 97:355–363.

Azadi, S., Klink, K.J., and Meade, B.J. 2004. Divergent immunological responses following glutaraldehyde exposure. *Toxicol. Appl. Pharmacol.* 197:1–8.

Basketter, D.A., Gilmour, N.J., Briggs, D., Ullmann, L.G., Gerberick, G.F., Ryan, C.A., Dearman, R.J., and Kimber, I. 2003. Utility of historical vehicle-control data in the interpretation of the local lymph node assay. *Contact Dermatitis* 49:37–41.

Basketter, D.A., Lea, L.J., Dickens, A., Briggs, D., Pate, I., Dearman, R.J., and Kimber, I. 1999. A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. *J. Appl. Toxicol.* 19:261–266.

Brown, K. 2008. High performance liquid chromatography identification, quantification, and fractionation of a suspect allergen, 4-chloro-3-methylphenol, in a LLNA-positive metalworking fluid. *Tribilogy Trans.* 51:1–6.

Cookson, J.O. (1971). Machine tool design and use in relation to cutting fluids. *Ann. Occup. Hyg.* 14:181–190.

de Boer, E.M., van Ketel, W.G., and Bruynzeel, D.P. 1989a. Dermatoses in metal workers. (I). Irritant contact dermatitis. *Contact Dermatitis* 20:212–218.

de Boer, E.M., van Ketel, W.G., and Bruynzeel, D.P. 1989b. Dermatoses in metal workers. (II). Allergic contact dermatitis. *Contact Dermatitis* 20:280–286.

Glaser, R., Pretty, J., Brown, K., Arnold, J., Lunsford, R.A., and Park, S.H. 2003. Analytical Method Research for Metalworking Fluids at the National Institute for Occupational Safety and Health (NIOSH). *Gefahrstoffe-Reinhaltung der Luft* 63:237–240.

Li, K., Aghazadeh, F., Hatipkarasulu, S., and Ray, T.G. 2003. Health risks from exposure to metal-working fluids in machining and grinding operations. *Int. J. Occup. Safety Ergo.* 9:75–95.

Menter, P., Harrison, W., and Woodlin, W.G. 1975. Patch testing of coolant fractions. *J. Occup. Med.* 17:565–568.

MMWR 2002. Respiratory illness in workers exposed to metalworking fluid contaminated with nontuberculous mycobacteria–Ohio, 2001. *MMWR Morb. Mortal. Wkly. Rep.* 51:349–352.

NIEHS (National Institute for Environmental Health Sciences) 1999. National Institute of Environmental Health Sciences (NIEHS); the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds. *Fed. Regist.* 64:14006–14007.

NIOSH (National Institute for Occupational Safety and Health) 1998a. *Criteria for a Recommended Standard: Occupational Exposure to Metalworking Fluids.* (P. H. S. U.S. Department of Health and Human Services, Center for Disease Control and Prevention, National Institute for Occupational Safety and Health, ed. DHHS. U.S. Department of Health and Human Services, Washington, DC.

NIOSH (National Institute for Occupational Safety and Health) 1998b. *What you need to know about occupational exposure to metalworking fluids.* DHHS Publication No. 98-116. U.S. Department of Health and Human Services, Washington, DC.

Pryce, D.W., Irvine, D., English, J.S., and Rycroft, R.J. 1989a. Soluble oil dermatitis: A follow-up study. *Contact Dermatitis* 21:28–35.

Pryce, D.W., White, J., English, J.S., and Rycroft, R.J. 1989b. Soluble oil dermatitis: A review. *J. Soc. Occup. Med.* 39:93–98.

Savonius, B., Keskinen, H., Tuppurainen, M., and Kanerva, L. 1994. Occupational asthma caused by ethanolamines. *Allergy* 49:877–881.

Shrank, A.B. 1985. Allergy to cutting oil. *Contact Dermatitis* 12:229.

Sloyer, J.L., Novitsky, T.J., and Nugent, S. 2002. Rapid bacterial counts in metal working fluids. *J. Ind. Microbiol. Biotech.* 29:323–324.

Sprince, N.L., Palmer, J.A., Popendorf, W., Thorne, P.S., Selim, M.I., Zwerling, C., and Miller, E.R. 1996. Dermatitis among automobile production machine operators exposed to metal-working fluids. *Am. J. Ind. Med.* 30:421–429.

Tapp, L., Ewers, L., Rodriguez, M., Jones, E., Tak, S., Brown, K.K., Shah, S., and Tucker, S.P. 2007. *Health Hazard Evaluation Report 2005-0227*. Diamond Chain Company National Institute for Occupational Safety and Health, Indianapolis, IN.

Thompson, D., Kriebel, D., Quinn, M.M., Wegman, D.H., and Eisen, E.A. 2005. Occupational exposure to metalworking fluids and risk of breast cancer among female autoworkers. *Am. J. Ind. Med.* 47:153–160.

USEPA (United States Environmental Protection Agency) 1990. *1990 HPV Challenge Program Chemical List.* U.S. Environmental Protection Agency, Washington, DC.

USEPA (United States Environmental Protection Agency) 1997. *R.E.D. FACTS: p-Chloro-m-cresol. Prevention, Pesticides and Toxic Substances.* EPA-783-F-96-008. U.S. Environmental Protection Agency, Washington, DC.

Verma, D.K., Shaw, D.S., Shaw, M.L., Julian, J.A., McCollin, S.A., and des Tombe, K. 2006. An evaluation of analytical methods, air sampling techniques, and airborne occupational exposure of metalworking fluids. *J. Occup. Environ. Hyg.* 3:53–66.

Woolhiser, M.R., Hayes, B.B., and Meade, B.J. 1998. A combined murine local lymph node and irritancy assay to predict sensitization and irritancy potential of chemicals. *Toxicol. Meth.* 8:245–256.