A COMBINED MURINE LOCAL LYMPH NODE AND IRRITANCY ASSAY TO PREDICT SENSITIZATION AND IRRITANCY POTENTIAL OF CHEMICALS

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In an effort to establish a single, rapid screening procedure for the sensitization and irritancy potential of new chemicals, the parameters of a murine Local Lymph Node Assay and a mouse ear swelling irritancy assay were combined. To validate this assay, a range of chemical irritants and sensitizers were evaluated for their ability to elicit responses in B6C3F1 female mice. Chemicals were administered for four consecutive days to the dorsal and ventral surfaces of each ear. An increase in ear thickness served to predict irritancy, while [^{3}H]thymidine uptake by cervical draining lymph nodes suggested sensitization. All chemicals known to be potent chemical sensitizers (oxazolone, 2,4-dinitrofluorobenzene, toluene diisocyanate) produced a marked lymph node cell proliferation in this assay. Animals exposed to irritating agents (sodium lauryl sulfate, croton oil, tetradecane, nonanoic acid, and benzalkonium chloride) experienced a significant increase in ear swelling. In addition, these irritating agents elicited low-level lymphocyte proliferation. In cases where chemicals are considered to be both potent sensitizers and irritants (2,4-dinitrofluorobenzene, toluene diisocyanate, and benzalkonium chloride), robust increases in [^{3}H]thymidine incorporation and ear swelling were demonstrated. Results were dose-responsive for all chemicals tested. The combined LLNA/ear swelling assay appears to be a reliable predictor of sensitization and irritancy potential, since it identified the activity of all eight chemicals tested. The advantages of this approach include a further reduction in the number of animals and time required to screen chemicals for both irritancy and/or sensitization potential. Although this assay does not have the capacity to discriminate between nonspecific lymph node proliferation and weak sensitizing ability of strong irritants, the information gained by the irritation component of the assay provides additional information when evaluating the significance of low-level lymphocyte proliferation in the LLNA. With further validation this assay could be useful as a common screening tool in the research and development of new chemical products.

Keywords B6C3F1, benzalkonium chloride, chemical allergen, contact sensitivity, croton oil, DNFB, ear swelling, hypersensitivity, in vivo, irritant, LLNA, nonanoic acid, oxazolone, SLS, TDI, tetradecane, mouse

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Skin diseases are the second most common type of occupational disease, accounting for approximately 13% of all work-related diseases, with an estimated annual cost of $1 billion [1]. Asthma is the most frequently diagnosed occupational respiratory disease, with approximately 9 million workers exposed to chemical irritants or sensitizers known to be associated with asthma. Costs associated with occupational asthma are estimated at $400 million. Because exposure to chemicals constitute a major risk in the development of dermatitis and respiratory disorders (e.g., rhinoconjuctivitits, asthma, and anaphylaxis), there continues to be a need during product research and development for accurate, rapid, and cost-effective methods to predict irritancy and sensitization potential of chemicals.

Allergic contact dermatitis is a T-cell–mediated event that requires previous antigen exposure for sensitization of the immune system; sensitization generally requires multiple exposures. Once sensitization has occurred, subsequent antigen exposure can elicit an allergic response. Conversely, irritant responses do not require previous immune recognition and may be elicited upon primary exposure, although weaker irritants may require cumulative exposures before causing irritation. Additionally, IgE-mediated, type I hypersensitivity responses can be manifested as contact urticaria, offering yet another mechanism of chemical-induced dermatitis [2].

Historically, the guinea pig and rabbit have been the species of choice for testing a chemical's sensitizing and irritating potential. Guinea pig contact sensitization assays are costly and time-consuming and result in the subjective endpoints of erythema and edema, making reproducibility and discrimination between allergic and irritation reactions difficult [3]. Among efforts to establish alternative animal models, the murine Local Lymph Node Assay (LLNA) was developed to identify chemicals as potential human sensitizers [4–6]. Following topical application of chemicals, this assay measures [^{3}H]thymidine incorporation into draining lymph nodes during the induction phase of sensitization. The advantages of the murine LLNA include its relevant exposure (no occlusion or adjuvants), shorter duration, cost-effectiveness, objective quantitative endpoint, and decreased number of animals required per evaluation. The LLNA has undergone international validation studies [7–9] and is a sensitization protocol accepted by the Organization for Economic Cooperation and Development (1992). The LLNA is also under consideration by the Interagency Coordinating Committee on the Validation of Alternative Methods (1998).

A murine ear swelling irritancy assay, modified from the phototoxicity assay described by Gerberick et al. [10] and similar to assays performed by other researchers [11, 12], has been used in combination with

hypersensitivity assays as part of a National Toxicology Program protocol to screen chemicals for irritancy and sensitization potential [13]. The following studies have combined the parameters of a mouse ear swelling irritancy assay and the LLNA into a single assay. The advantages of this approach include a further reduction in the number of animals and time required to screen chemicals for both irritancy and sensitization potential. This assay does not have the capacity to discriminate nonspecific lymph node proliferation from the weak sensitizing ability of strong irritants. However, the information gained by the irritation component of the assay provides additional information when the significance of low-level lymphocyte proliferation in the LLNA is being evaluated. To begin validating this approach, eight common irritants and sensitizers were evaluated.

MATERIALS AND METHODS

Animals

Female B6C3F1 mice (C57BL/6 × C3HHeN) were purchased from Taconic Farms (Germantown, NY). Upon arrival, the mice were quarantined for 1 week prior to use. Mice were weighed and assigned to homogeneous weight groups ($n = 5$). Animals weighed 18–26 g at study start and were maintained on Agway Rat and Mouse Ration (NIH 07) and tap water ad libitum. Mice were maintained under conditions specified within NIH guidelines. Animal rooms were maintained, between 18 and 26°C and 40–70% relative humidity with light/dark cycles of 12-h intervals.

Chemicals

4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone; purity ≥90%), 2,4-dinitrofluorobenzene (DNFB; purity ≥99%), sodium lauryl sulfate (SLS; purity ≥95%), benzalkonium chloride, croton oil, nonanoic acid (pelargonic acid; purity ≥97%), toluene diisocyanate (TDI; purity 99.6%), tetradecane (purity ≥99%), and acetone were all purchased from Sigma Chemical (St. Louis, MO). All chemicals were prepared in acetone except for SLS, which was dissolved in 30% ethanol (Fischer Scientific, Columbia, MD). Concentrations tested are shown in Table 1.

LLNA/Irritancy Combined Assay

The LLNA and irritancy ear swelling procedures combined for this assay were modified from those described by Hayes et al. [13]. Prior to

TABLE 1. Experimental Groups

Chemical	Vehicle	Percent concentration				
Oxazolone (v/v)[a]	Acetone	0.00125	0.0025	0.005	0.01	0.025
DNFB (v/v)	Acetone	0.025	0.05	0.10	0.15	0.20
SLS (w/v)[b]	30% Ethanol	5.0	10.0	20.0	30.0	40.0
Benzalkonium chloride (v/v)	Acetone	0.5	1.0	2.0	3.0	5.0
Croton oil (w/v)	Acetone	0.025	0.05	0.1	0.25	0.5
TDI (v/v)	Acetone	0.1	0.5	1.0	2.5	—
Tetradecane (v/v)	Acetone	20.0	30.0	40.0	50.0	—
Nonanoic acid (v/v)	Acetone	5.0	10.0	20.0	40.0	60.0

[a] Percent concentration prepared as a volume:volume ratio.
[b] Percent concentration prepared as a weight:volume ratio.

exposures, right and left ear measurements were taken in duplicate using a modified Mitutoyo micrometer (Mitutoyo, Japan) and averaged to give a baseline ear thickness for each ear. On days 1–4, vehicle or test solutions were administered topically to the dorsal and ventral surfaces of each ear (12.5 μL on each side for a total of 50 μL/mouse day^{-1}). All exposures were accomplished ± 2 h from the previous day. On day 5, each animal was intravenously injected via one of the lateral tail veins with 0.2 mL phosphate-buffered saline (PBS) containing 20 μCi [^{3}H]thymidine (Dupont NEN; specific activity 2 Ci/mmol). Following intravenous injection and approximately 24 h after the final chemical application, post-treatment ear measurements were taken in duplicate and averaged for each ear. Irritation was indicated by percent increase in ear thickness and was calculated for each ear as follows: % change in ear swelling = (post-treatment ear thickness/pretreatment ear thickness) × 100 − 100.

Five hours after [^{3}H]thymidine injection, animals were euthanized by carbon dioxide asphyxiation. Right and left cervical draining lymph nodes located near the bifurcation of the jugular vein were excised and pooled for each animal. Lymph nodes were prepared as single-cell suspensions for each animal by mechanical disaggregation using frosted microscope slides. The samples were washed twice with PBS and precipitated in 5% trichloroacetic acid (TCA) overnight. The following day, cell suspensions were pelleted (200*g* for 10 min), resuspended in 1 mL TCA, and transferred to 5 mL of scintillation cocktail. Test tubes were rinsed with an additional 1 mL TCA and added to scintillation vials. [^{3}H]thymidine incorporation was determined via beta liquid scintillation counting (LKB Wallac 1218 Rack Beta Liquid Scintillation Counter) and disintegrations per minute for each mouse (dpm/2 nodes) were calculated.

Statistics

Body weights, percent ear swelling, and lymph node proliferation data were analyzed by one-way analysis of variance (ANOVA). When significant differences were detected ($p < 0.05$), test groups were compared to vehicle groups using Dunnett's test [14].

RESULTS

With the exception of erythema and edema of the ears resulting from chemical exposure, all animals appeared clinically normal throughout the study. No changes in body weights were seen between test and vehicle groups, with the exception of animals in the high-dose benzalkonium-chloride–exposed group (data not shown).

Oxazolone caused significant increases in lymph node proliferation at 0.01 and 0.025% but demonstrated no irritancy potential as measured by ear swelling (Figure 1). Exposure to DNFB resulted in a dose-responsive lymph node proliferation with a peak response occurring at 0.15% (Figure 2). The strong irritating potential of DNFB was demonstrated by a dose-dependent increase in ear swelling culminating in greater than 160% swelling at a concentration of 0.20%. Similarly, TDI elicited dose-responsive increases in lymph node proliferation and ear swelling at concentrations up to and including 2.5% (Figure 3).

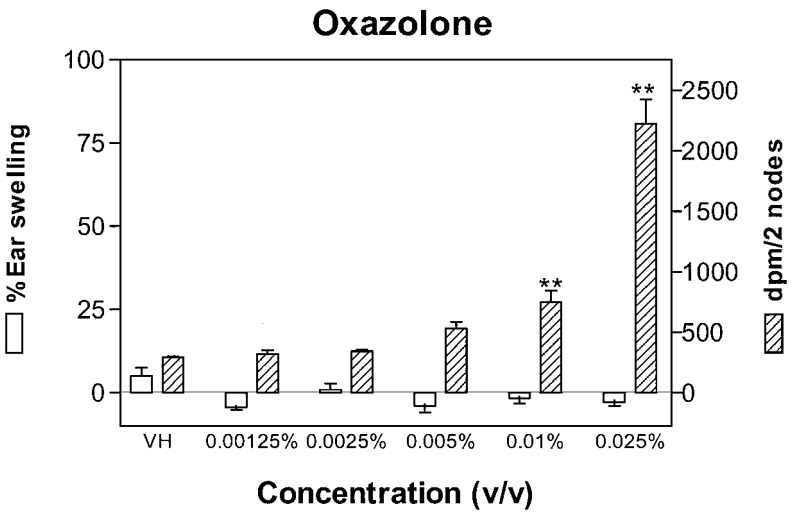

FIGURE 1. Ear swelling and lymph node cell proliferation following oxazolone exposure. Data are expressed as group means ± standard error; $n = 5$ mice/group. Double asterisks indicate statistical significance from vehicle controls (VH) at $\alpha = 0.01$ using Dunnett's test.

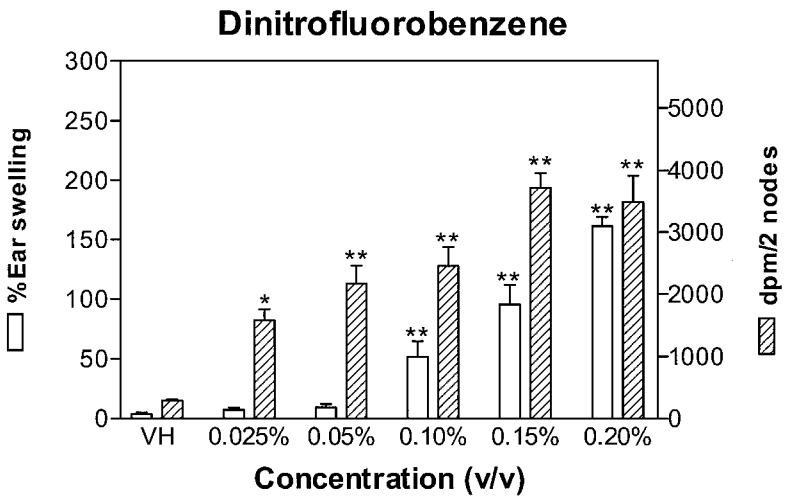

FIGURE 2. Ear swelling and lymph node cell proliferation following DNFB exposure. Data are expressed as group means ± standard error; $n = 5$ mice/group. Asterisks indicate statistical significance from vehicle controls (VH) at $\alpha = 0.05$ (single asterisk) or $\alpha = 0.01$ (double asterisk) using Dunnett's test.

The strong irritant croton oil elicited pronounced elevations in ear swelling at all concentrations tested with a dose-responsive increase up to 0.25% (Figure 4). A dose-dependent increase in lymphocyte proliferation was seen up to and including 0.1% croton oil and reached a plateau at a level nearly 6-fold greater than vehicle-treated mice. Tetradecane exposure resulted in significant irritation as evidenced by

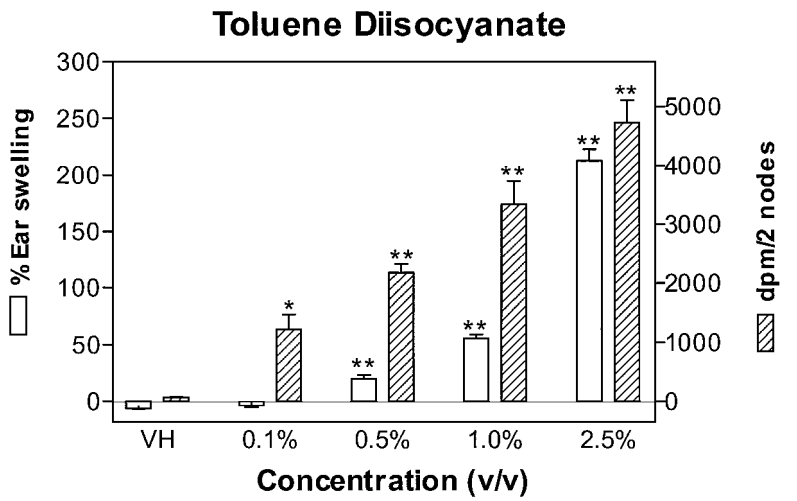

FIGURE 3. Ear swelling and lymph node cell proliferation following TDI exposure. Data are expressed as group means ± standard error; $n = 5$ mice/group. Asterisks indicate statistical significance from vehicle controls (VH) at $\alpha = 0.05$ (single asterisk) or $\alpha = 0.01$ (double asterisk) using Dunnett's test.

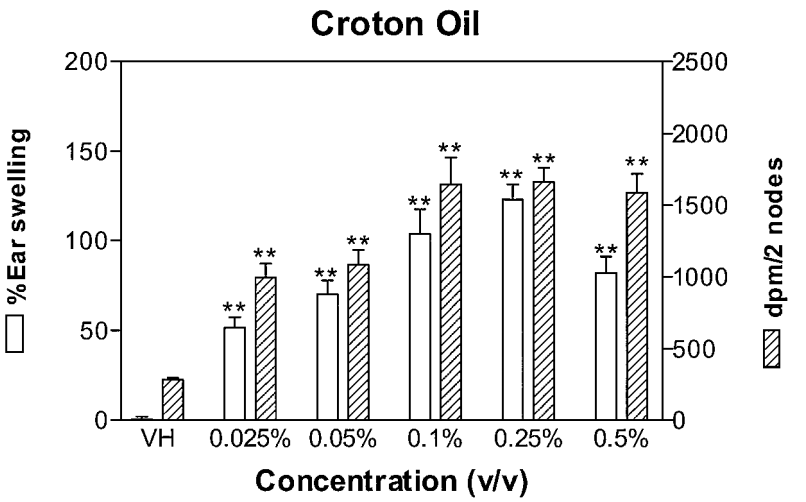

FIGURE 4. Ear swelling and lymph node cell proliferation following croton oil exposure. Data are expressed as group means ± standard error; $n = 5$ mice/group. Double asterisks indicate statistical significance from vehicle controls (VH) at $\alpha = 0.01$ using Dunnett's test.

dose-responsive ear swelling (Figure 5). Low but statistically significant increases in lymph node proliferation occurred following 30, 40, and 50% tetradecane treatment. Benzalkonium chloride elicited dose-responsive irritation that peaked at 5%, resulting in greater than 114% ear swelling (Figure 6). Lymph node proliferation in these animals peaked following 2% benzalkonium chloride applications and fell to nonsignificant levels

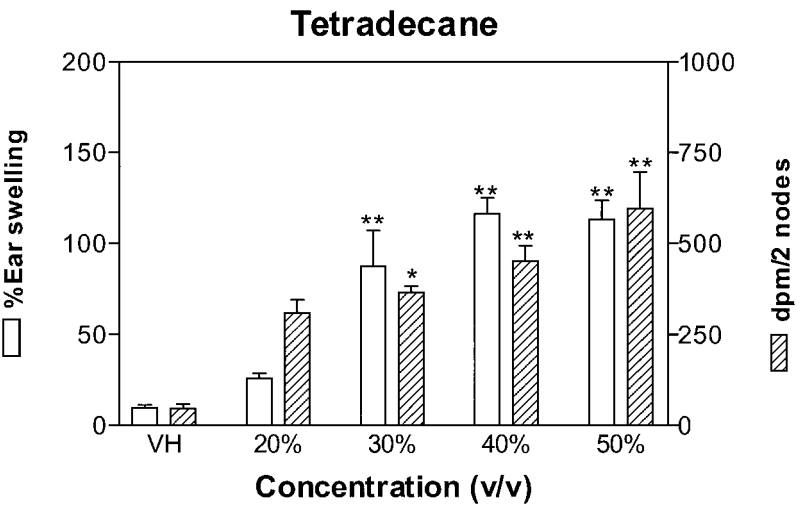

FIGURE 5. Ear swelling and lymph node cell proliferation following tetradecane exposure. Data are expressed as group means ± standard error; $n = 5$ mice/group. Asterisks indicate statistical significance from vehicle controls (VH) at $\alpha = 0.05$ (single asterisk) or $\alpha = 0.01$ (double asterisk) using Dunnett's test.

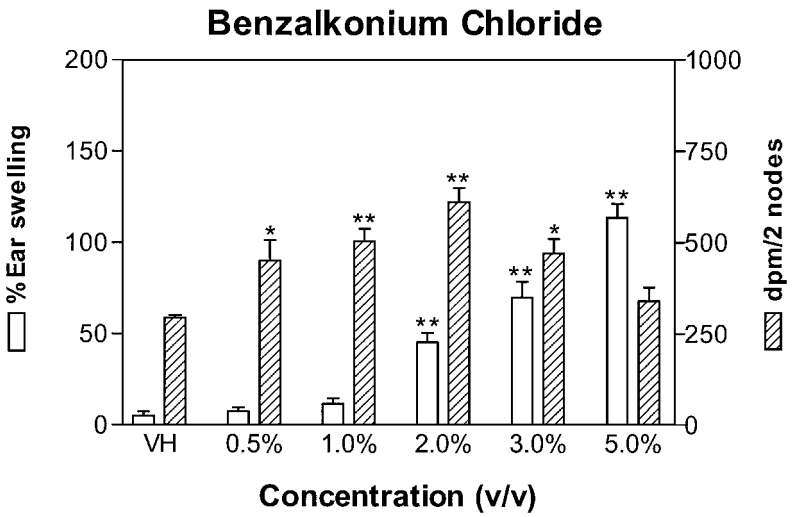

FIGURE 6. Ear swelling and lymph node cell proliferation following benzalkonium chloride exposure. Data are expressed as group means ± standard error; $n = 5$ mice/group. Asterisks indicate statistical significance from vehicle controls (VH) at $\alpha = 0.05$ (single asterisk) or $\alpha = 0.01$ (double asterisk) using Dunnett's test.

at a concentration of 5%. The fall in proliferative response following exposure to 5% benzalkonium chloride coincided with a 19.7% body weight loss, suggesting toxicity at this dose level.

The irritants SLS and nonanoic acid elicited dose-dependent increases in ear swelling with no effect levels at 10% (Figures 7 and 8).

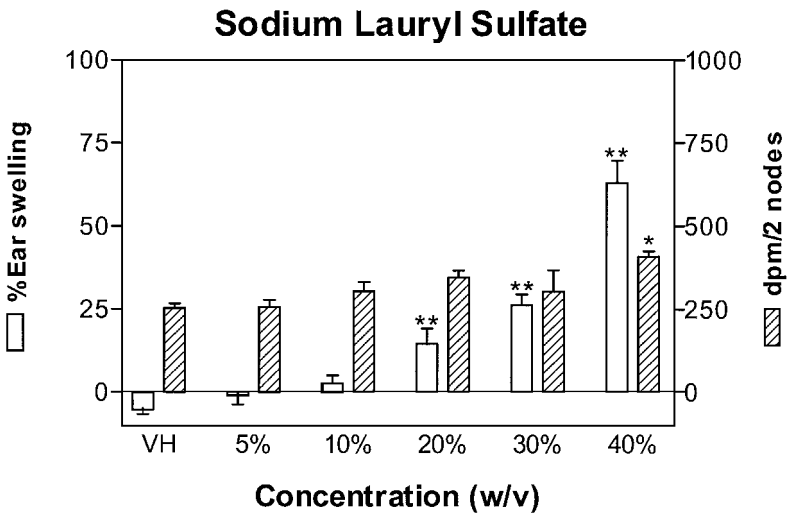

FIGURE 7. Ear swelling and lymph node cell proliferation following SLS exposure. Data are expressed as group means ± standard error; $n = 5$ mice/group. Asterisks indicate statistical significance from vehicle controls (VH) at $\alpha = 0.05$ (single asterisk) or $\alpha = 0.01$ (double asterisk) using Dunnett's test.

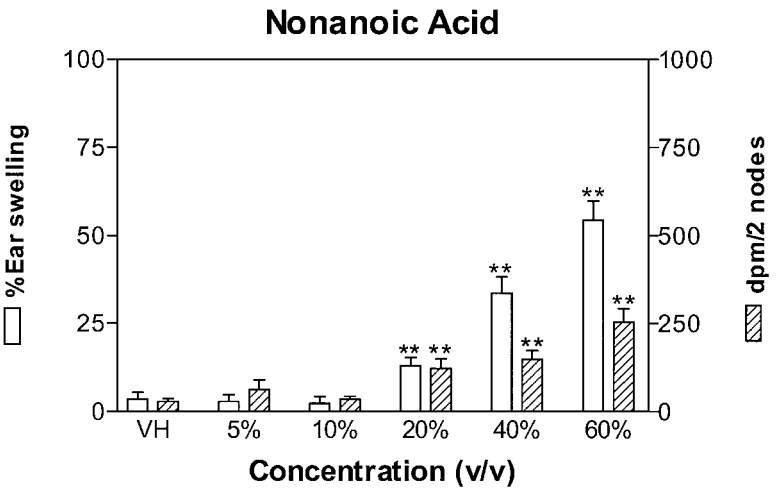

FIGURE 8. Ear swelling and lymph node cell proliferation following nonanoic acid exposure. Data are expressed as group means ± standard error; $n = 5$ mice/group. Double asterisks indicate statistical significance from vehicle controls (VH) at $\alpha = 0.01$ using Dunnett's test.

A low but statistically significant lymph node proliferation was seen in animals exposed to 40% SLS (Figure 7). Nonanoic acid elicited dose-dependent and statistically significant increases in lymph node proliferation, although maximum proliferation was not above 250 dpm (Figure 8).

DISCUSSION

The chemical allergens DNFB, TDI, and oxazolone were identified as potent sensitizers using the combined assay. In addition, elevations in ear thickness following exposure to DNFB and TDI were consistent with their known irritation potential. The chemical irritants SLS, croton oil, tetradecane, benzalkonium chloride, and nonanoic acid all resulted in significant ear swelling following topical application to the ears. The irritancy potential of SLS in humans has repeatedly been reported [15–20]. Nonanoic acid, croton oil, and benzalkonium chloride have also been identified as irritants following human exposure [15, 16, 19, 21, 22]. Literature regarding the irritancy potential of tetradecane, and other *n*-alkanes, in humans is limited; Moloney and Teal reported ear edema following application to the ears of mice with 50% tetradecane [23].

The LLNA does not have the capacity to discriminate between non-specific proliferation and weak sensitizing ability of stronger irritants. SLS elicited a weak but statistically significant positive lymph node response at the highest concentration tested (40%). Although a few case reports suggesting SLS contact sensitivity have been published [24, 25],

SLS is considered an irritant by most researchers and dermatologists. Minimal increases in lymph node proliferation were also seen following exposure to tetradecane and nonanoic acid. Croton oil, which produced severe irritation, induced levels of lymph node proliferation consistent with that seen for contact sensitizers. No literature was found, however, regarding the sensitization potential of these three irritants (tetradecane, nonanoic acid, and croton oil) in humans. Benzalkonium chloride elicited low, but dose-responsive and statistically significant lymph node proliferation at concentrations that did not induce measurable irritation. Furthermore, benzalkonium chloride has been reported to be a contact sensitizer [26–31].

It is hypothesized that minimal lymph node proliferation may occur following irritant exposure due to nonspecific Langerhans cell migration to draining lymph nodes. Irritant exposure can also result in necrosis, keratinocyte damage, mononuclear infiltrate [16], and/or stratum corneum protein damage, which alters the skin barrier [32]. Furthermore, irritation can compromise cutaneous barriers leading to TNF, IL-1, and GM-CSF elevations [33]. In keratinocyte cultures, irritants have induced cytokines such as IL-1α, TNFα, IL-8, and GM-CSF [34]. These cytokines can act as chemokines and inducers of immune cell function. Definitive differentiation of weak allergens from chemical irritants may ultimately depend on a combination of endpoints, including cytokine profiles or lymphocyte phenotyping.

The combined assay has been successfully used in this laboratory to test both complete formulations of chemical products and individual components to identify ingredients responsible for adverse responses. This methodology can reduce animal usage, time, and therefore costs approximately 50% by testing irritation and sensitization potential simultaneously. Furthermore, simultaneous evaluation of irritation and sensitization may provide insight when interpreting low-level lymph node proliferation following chemical exposure. The combined sensitization/irritancy assay appears to be a reliable predictor of sensitization and irritancy potential, since it identified the expected activity for all eight chemicals tested. With further validation, this assay could be used as a rapid preliminary screening tool in the research and development of new chemical products.

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