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**CONTACT HYPERSENSITIVITY TO DICYCLOHEXYLCARBODIIMIDE
AND DIISOPROPYLCARBODIIMIDE IN FEMALE B6C3F1 MICE**

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ABSTRACT

Dicyclohexylcarbodiimide (DCC) and diisopropylcarbodiimide (DIC) are two commonly used coupling reagents in protein synthesis resulting in exposure of individuals in chemical and pharmaceutical industries as well as research laboratories involved in protein synthesis and recombinant DNA techniques. The objectives of these studies were to determine the irritation and sensitizing potential of these two compounds when applied topically to B6C3F1 mice. Sensitization potential was assessed by the Mouse Ear Swelling Test (MEST) and the murine Local Lymph Node Assay (LLNA). Concentrations used in the contact hypersensitivity assays were determined by primary irritancy studies. DCC and DIC were identified as both irritants and contact sensitizers with the MEST being a more sensitive indicator of sensitization potential. The MEST identified DCC as a sensitizer at concentrations as low as 0.006% (w/v) 24 hr and 48 hr post challenge and DIC at 0.3% (w/v) and 1.5% (w/v) 24 and 48 hr post challenge, respectively. In the LLNA, the lowest concentrations yielding a significant response were 0.06% (w/v) for DCC and 10% (w/v) for DIC.

INTRODUCTION

Allergic contact dermatitis (ACD) is a common skin disease usually associated with pruritus, erythema, edema, and skin lesions. The ACD condition is a T-cell mediated immune response against a xenobiotic that requires prior sensitization. This sensitization process can be separated into three general categories: a) antigen penetrating the skin's horny layer into the viable epidermis b) Langerhans cells processing the antigen (e.g. hapten-protein complex) and migration to local lymph nodes c) antigen presentation and T-cell sensitization and proliferation.

After sensitization, subsequent antigen exposure can elicit the characteristic inflammatory response involving both "arms" of the immune system, innate and adaptive.

Historically, the first contact hypersensitivity studies were done on humans¹ and then guinea pig models^{2,3} emerged as the paradigm for hypersensitivity testing. In the last few decades, the mouse has been investigated as an alternative model for evaluating cutaneous hypersensitivity responses with good success.⁴⁻⁸ With regard to immune parameters, the mouse has been studied extensively and is an attractive model both from a time and an economical standpoint. The current National Toxicology Program (NTP) hypersensitivity testing protocol is based on several factors which have been reported to influence the development of contact sensitization in animal models. Klecak *et al.*⁹ demonstrated that epicutaneous administration of chemicals could elicit hypersensitivity responses equivalent to intradermal injection. In addition, Gad *et al.*¹⁰ proposed using ear thickness for identifying allergic responses in the Mouse Ear Swelling Test (MEST). Finally, Kimber *et al.*^{11,12} demonstrated that [³H]thymidine uptake could be used as a predictive test for contact allergens and developed the murine Local Lymph Node Assay (LLNA) as an alternative mouse assay for determining the sensitization potential of xenobiotics.

Dicyclohexylcarbodiimide (DCC) and diisopropylcarbodiimide (DIC) are two commonly used coupling reagents in protein syntheses¹³ resulting in exposure of individuals in chemical and pharmaceutical industries as well as research laboratories involved in protein synthesis and recombinant DNA techniques. However, only limited numbers of allergic contact reactions to carbodiimides have been reported since the mid-1970's.¹⁴⁻²¹ Thus, DCC and DIC were nominated for evaluation by the NTP immunotoxicology testing program, and the following studies were aimed at assessing the irritation and sensitization potential of DCC and DIC in murine models of hypersensitivity.

MATERIALS AND METHODS

Test Articles

Dicyclohexylcarbodiimide (mw 206.3) and diisopropylcarbodiimide (mw 126.2) were obtained from Aldrich Chemical Company (Milwaukee, WI) with a lot purity of 99% (Fig 1). Both chemicals are moisture sensitive and were refrigerated in a sealed desiccator until used. Acetone (99.8% purity, Sigma Chemical, St. Louis MO) was used as the vehicle for all concentrations and each test article was prepared daily prior to dosing animals.

Positive control

1-Fluoro-2,4-dinitrobenzene (DNFB, Sigma Chemical and ICN Pharmaceuticals) was used as the positive control for the MEST and LLNA. DNFB is light sensitive and was prepared daily by dilution in acetone and stored in amber vials. Sensitization concentrations were 0.25% (v/v) for the MEST and the LLNA and 0.5% (v/v) for challenge in the MEST.

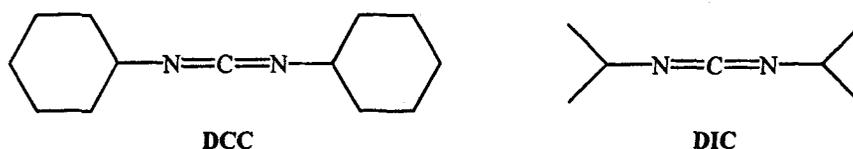


FIGURE 1

Structures of Dicyclohexylcarbodiimide and Diisopropylcarbodiimide

Animals

Female B6C3F1 mice (4-5 weeks, 17-20 grams) were purchased from Taconic Farms (Germantown, NY) hepatitis and Sendai virus free and quarantined until 8 weeks of age. The mice were maintained on Agway Rat and Mouse Ration (NIH 031) and tap water *ad libitum*. The animal facility maintained a temperature between 18-26°C, a relative humidity between 40-70%, and a light/dark cycle of 12-hour intervals throughout the experimental period.

Irritancy Assay

A primary irritancy study on the carbodiimides was conducted to establish sensitization and challenge concentrations and to ensure the mice would tolerate multiple days of topical exposure. This assay was a modification of the phototoxicity murine model developed by Gerberick and Ryan.²² Prior to dosing on the first day, measurements of ear thickness were made in duplicate with a modified Mitutoyo micrometer (Mitutoyo Corporation, Japan), recorded in mm x 10⁻², and averaged. With a pipette, 12.5 µl of the test material or vehicle was applied to both sides of each ear pinna for four consecutive days (50 µl/mouse/day). Twenty-four hours (±2 hr) after the final treatment, mice were restrained and ear thickness was measured in the identical manner as pretreatment measurements. Percent ear swelling was calculated as follows: [(mean post-treatment ear thickness / mean pre-treatment ear thickness) x 100]-100. The mean percent ear swelling for each treatment group was calculated and compared to the vehicle control for statistical significance and dose response. The highest concentration producing a percent ear swelling not significantly different from the vehicle was considered the Maximal Non-Irritating Concentration (MNC). The lowest concentration of test article producing a percent ear swelling significantly greater than the vehicle was considered the Minimal Irritating Concentration (MIC). Initial concentrations of DCC tested were 0.001, 0.005, 0.01, 0.1%. When no significant irritation was found, subsequent studies tested concentrations of DCC up to 30%. Two irritancy studies were conducted for DIC with concentrations ranging from 0.06% to 20%.

Contact Hypersensitivity Assays

The Mouse Ear Swelling Test (MEST) followed the original procedure described by Gad *et al*¹⁰

TABLE I
Concentrations Tested in Hypersensitivity Assays

DCC		DIC	
MEST	LLNA	MEST	LLNA
VH	VH	VH	VH
BC	0.006%	BC	1.5%
0.003%	0.03%	0.03%	3.0%
0.006%	0.06%	0.15%	10.0%
0.03%	0.1%	0.3%	----
0.06%	----	1.5%	----
----	----	3.0%	----

Percentages are in Wt/Vol.

with only minor modifications. Prior to dosing, mice were weighed, tattooed, and anesthetized so the dorsal lumbar area of each mouse could be shaved. Initial concentrations of DCC and DIC evaluated were the 0.1MNC, 0.5MNC, and MNC. Lower concentrations were tested when necessary to elicit a no-effect level (Table I). Beginning on day 1, 50 μ l of test article or vehicle was applied to the shaved site of each animal. The same procedures were repeated for two more days and then the mice were left untreated for four days (days 4-7). On day 8, right ear thickness of each mouse was measured in duplicate and averaged prior to challenge. After measurement, mice were challenged with 12.5 μ l of vehicle or test article (MIC concentration) on the dorsal and ventral surfaces of the right ear pinna. The right ear then was measured in duplicate 24 and 48 hours (\pm 2 hr) after challenge and averaged. The percent ear swelling was calculated for each time point as described for the irritancy assay. Mean percent ear swelling for each dose group was compared to the mean percent ear swelling for the background control (BC, sensitization with acetone and challenge with MIC) for significance and dose response. The BC group was used as the control to account for swelling caused by irritation.

The Local Lymph Node Assay (LLNA) used for these studies was a modification of the procedure developed by Kimber et al.²³ Doses chosen for testing in the LLNA were the 0.5MNC, MNC, and MIC. Lower concentrations were tested when necessary to elicit a no-effect level (Table I). On days 1-3, mice were treated with 12.5 μ l of test article or vehicle on both the dorsal and the ventral surfaces of each ear. Animals were left untreated on day 4. On day 5, mice were injected intravenously via tail vein with 0.2 ml (20 μ Ci) [³H]thymidine. Approximately 5 hours after injection, mice were euthanized with CO₂ gas and cervical draining

lymph nodes located at the bifurcation of the jugular veins were excised. Right and left lymph nodes from each animal were pooled and a single cell suspension was prepared by gentle disaggregation with frosted microscope slides. Cells were washed twice in 10 ml of PBS and then precipitated in 3 ml of 5% trichloroacetic acid (TCA). Following overnight incubation at 4° C, the precipitates were pelleted, resuspended in 1 ml TCA, and transferred to 5 ml of scintillation solution. [³H]Thymidine incorporation was quantified with a LKB Wallac 1218 Beta counter (Turku, Finland) with an efficiency of 63.6% for [³H]thymidine. Mean cpm for each group was calculated and compared to the vehicle control for significance and dose response.

Safety

All personnel handling potentially sensitizing chemicals wore protective clothing including lab coats, latex gloves, and masks. Chemicals were diluted and animals exposed under a chemical fume hood.

Statistical Analysis

The data were first tested for homogeneity by the Bartlett's Chi Square Test.²⁴ If homogeneous, a one-way analysis of variance (ANOVA) was conducted.²⁵ If the ANOVA showed significance at $p < 0.05$ or less, the Dunnett's Test²⁶ was used to compare treatment groups with the appropriate control group. If data were non-homogeneous, a non-parametric analysis of variance²⁷ and a Wilcoxon's Rank Sum Test²⁸ were used to compare treatment groups with the appropriate control groups. The Jonckheere's Test was used to detect trend analysis and dose dependency.²⁹

RESULTS

Irritancy Study

Exposure to concentrations higher than 3% DCC resulted in significant toxicity. No gross visible lesions were seen in these animals. Animals exposed to 3% DCC experienced significant weight loss and presented with ataxia, hunched stance, and squinting eyes. In the DIC study, all animals appeared clinically normal throughout the experimental period with the exception of the high dose group (20%). These animals experienced mild ataxia and generalized weakness after 3 days of dosing. Within minutes of receiving the fourth dose, the animals became severely dyspneic, were agonal within one hour, and were euthanized. Necropsy revealed pulmonary hemorrhage in the caudal lobes of one animal and all animals in the group had mild hemothorax. There were no statistically significant body weight changes at any concentrations of DIC tested (data not shown).

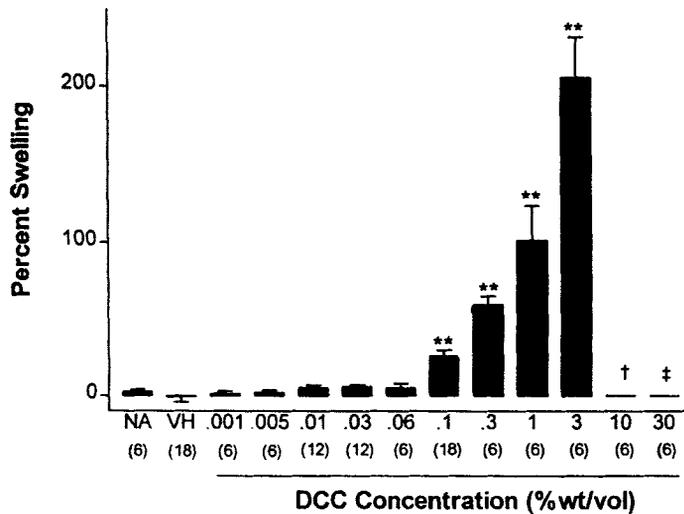


FIGURE 2

Primary Irritancy Response to Dicyclohexylcarbodiimide (DCC) in Female B6C3F1 Mice

Bars represent means \pm SE of the number of samples indicated in parentheses. Dose groups were determined to be nonhomogenous (Bartlett's Test) and compared to the vehicle control group by the Wilcoxon Test where ** represents $p < 0.01$. Trend analysis was found to be significant at $p < 0.01$ by the Jonckheere's Test. "†" denotes deceased animals on day 2 of the study, "‡" denotes deceased animals on day 3 of the study. NA=naive and VH=vehicle.

Animals exposed to DCC and DIC experienced dose dependent irritation responses (Fig 2 and 3). For DCC, the MNC and MIC were found to be 0.06% and 0.1%, respectively and for DIC, the MNC was 3% and the MIC was 10%.

Mouse Ear Swelling Test

Dose dependent contact hypersensitivity responses to both DCC and DIC were demonstrated by the MEST. Sensitizing concentrations as low as 0.006% DCC produced significant ear swelling 24 and 48 hr after application compared to the background control group (Fig 4). Similarly, sensitization with concentrations as low as 0.3% DIC produced a significant increase in percent ear swelling 24 hrs post challenge. By 48 hrs post challenge, 1.5% DIC was the lowest concentration producing a statistically significant response (Fig 5). No significant differences were seen in body weight changes between animals dosed with vehicle or test article in any of the MEST studies. The positive control groups experienced a significant increase in percent ear swelling as compared to the background positive at both the 24 and 48 hr time points (representative studies are shown in Figs 4 and 5).

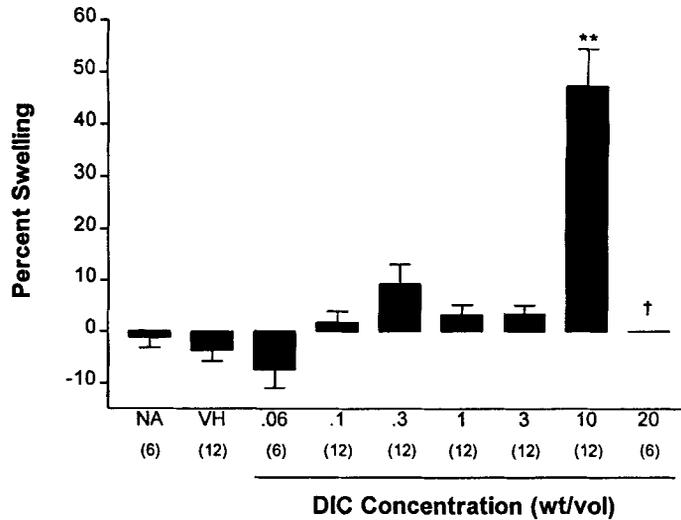


FIGURE 3

Primary Irritancy Response to Diisopropylcarbodiimide (DIC) in Female B6C3F1 Mice

Bars represent means \pm SE of the number of samples indicated in parentheses. Dose groups were determined to be nonhomogenous (Bartlett's Test) and compared to the vehicle control group by the Wilcoxon Test where ** represents $p < 0.01$ as compared to VH. Trend analysis was found to be significant at $p < 0.01$ by the Jonckheere's Test. "†" represents moribund animals removed on day 4 of the study. NA=naive and VH=vehicle.

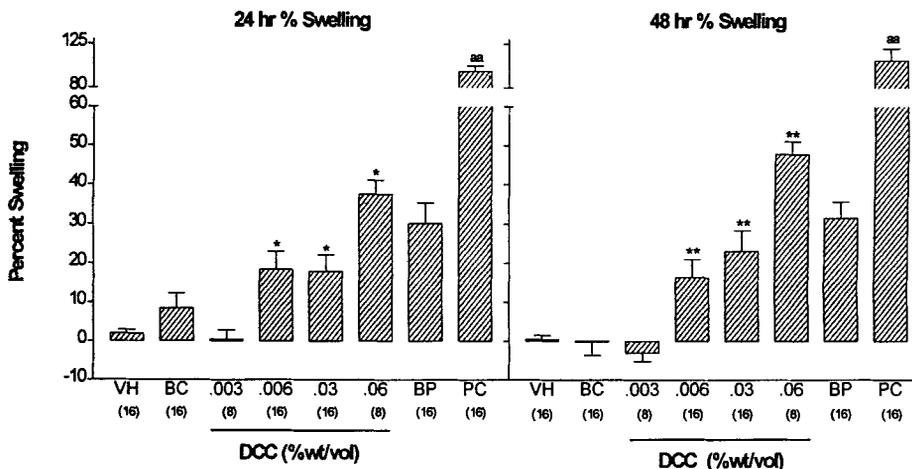


FIGURE 4

MEST Response to Dicyclohexylcarbodiimide (DCC) in Female B6C3F1 Mice

Bars represent means \pm SE of the number of animals indicated in parentheses. Dose groups were determined to be nonhomogenous (Bartlett's Test) and compared to the BC (sensitized with acetone vehicle and challenged with 0.1% DCC) by the Wilcoxon Test where * represents $p < 0.05$ and ** represents $p < 0.01$. The PC group (sensitized with 0.25% DNFB and challenged with 0.5% DNFB) was compared to the BC (sensitized with acetone vehicle and challenged with 0.5% DNFB) for significance where "aa" represents $p < 0.01$. Trend analysis was found to be significant at $p < 0.01$ by the Jonckheere's Test. NA=naive, VH=vehicle, BC=background control, and PC=positive control.

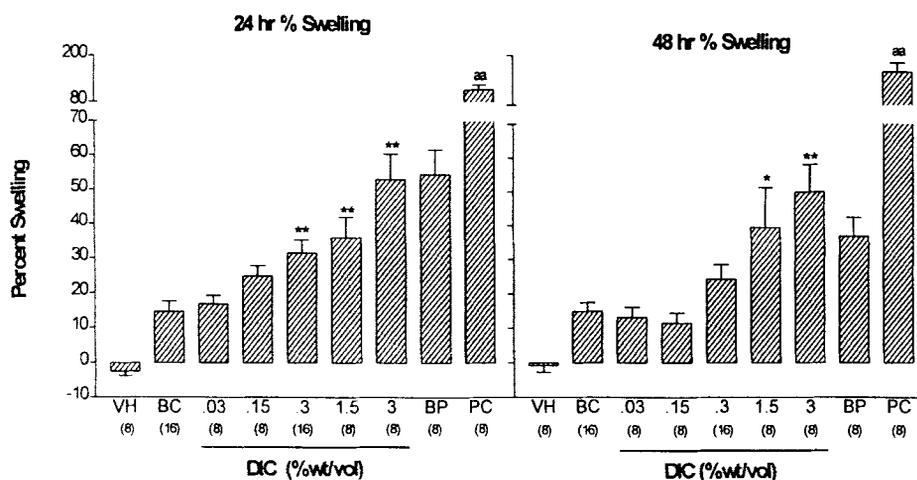


FIGURE 5

MEST Response to Diisopropylcarbodiimide (DIC) in Female B6C3F1 Mice

Bars represent means \pm SE of the number of animals indicated in parentheses. Twenty-four hour data points were determined to be homogenous (Bartlett's Test) and compared to the BC (sensitized with acetone vehicle and challenged with 0.1% DCC) using the Dunnett's test where ** represents $p < 0.01$ as (10% DIC). Forty-eight hour data points were determined to be nonhomogenous (Bartlett's Test) and compared to the BC by the Wilcoxon Test where * represents $p < 0.05$ and ** represents $p < 0.01$. The PC group (sensitized with 0.25% DNFB and challenged with 0.5% DNFB) was compared to the BC (sensitized with acetone vehicle and challenged with 0.5% DNFB) for significance where "aa" represents $p < 0.01$. Trend analysis was found to be significant at $p < 0.01$ by the Jonckheere's Test. NA=naive, VH=vehicle, BC=background control, and PC=positive control.

Local Lymph Node Assay

A dose dependent increase in lymph node cell proliferation was detected with the LLNA for both carbodiimides. DCC produced a significant increase in lymph node proliferation at concentrations of 0.06% and 0.1% (Fig 6). The lowest concentration of DIC inducing a significant response was 10% DIC (Fig 7). The positive control groups experienced a significant increase in lymph node cell proliferation as compared to control (representative studies are shown in Figs 6 and 7).

DISCUSSION

Information on human sensitization to the carbodiimides is limited to a small number of case reports and no published data are available on human exposure levels. Zschunke and Folesky¹⁴ reported that a concentration of 1% DCC was toxic and recommended 0.1% DCC in acetone for patch testing. This recommendation is consistent with murine models in which 0.1% DCC was

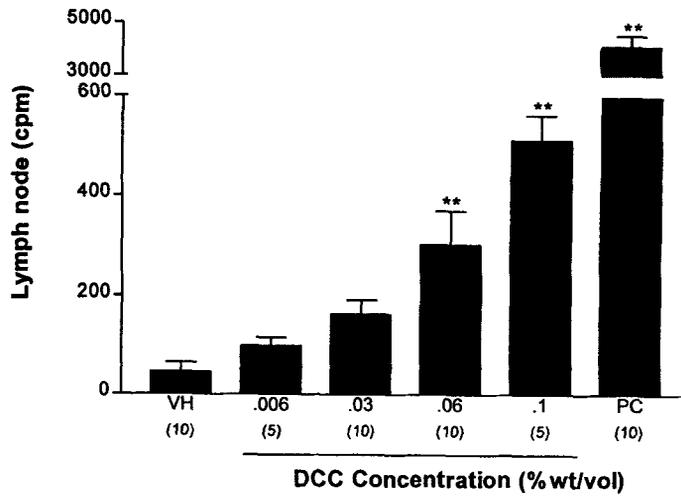


FIGURE 6

LLNA Response to Dicyclohexylcarbodiimide (DCC) in Female B6C3F1 Mice

Bars represent means \pm SE of the number of animals shown in parentheses. Dose groups were determined to be nonhomogenous (Bartlett's Test) and compared to the vehicle control group by the Wilcoxon Test where ** represents $p < 0.01$. Trend analysis was found to be significant at $p < 0.01$ by the Jonckheere's Test. Lymph node (cpm)=sum of cpm for left and right draining lymph nodes. VH=vehicle and PC=positive control (0.25% DNFB).

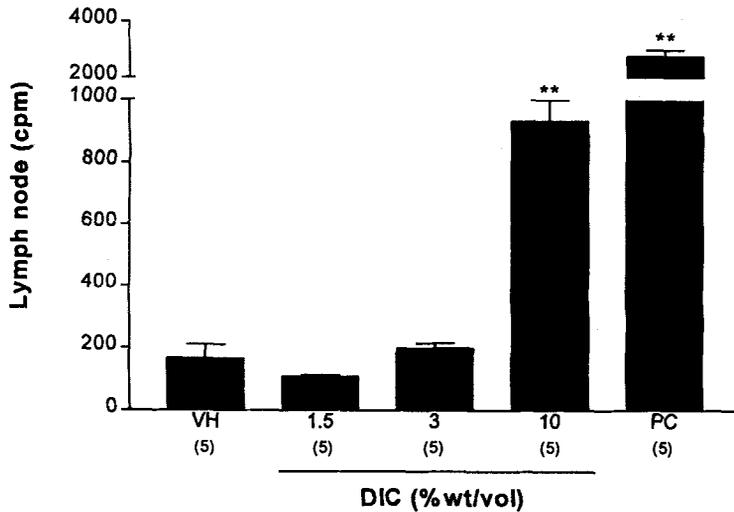


FIGURE 7

LLNA Response to Diisopropylcarbodiimide (DIC) in Female B6C3F1 Mice

Bars represent means \pm SE of the number of animals samples indicated in parentheses. Dose groups were determined to be nonhomogenous (Bartlett's Test) and compared to the vehicle control group by the Wilcoxon Test where ** represents $p < 0.01$. Trend analysis was found to be significant at $p < 0.01$ by the Jonckheere's Test. Lymph node (cpm)=sum of cpm for left and right draining lymph nodes. VH=vehicle and PC=positive control (0.25% DNFB).

determined to be the MIC and was chosen for the challenge dose in the MEST in order to produce an optimal response. Other investigators were able to elicit a positive response in DCC sensitized individuals at patch test concentrations of 0.05%²⁰ and 0.0125%.²¹ For DIC, 10% was found to be the MIC in the marine irritancy model and concentrations as low as 0.05% have been shown to elicit a positive patch test response in humans.²⁰ Results from these studies demonstrate strong correlations between the concentrations required for elicitation of hypersensitivity responses in murine models and humans. Although no published data are available on human exposure or concentrations required for sensitization, these studies indicate that in murine models concentrations as low as 0.006% DCC and 0.3% DIC are capable of inducing sensitization.

DCC was found to be a more potent irritant and sensitizer than DIC. Structure activity analysis has shown that nitrogen-carbon biophores are highly reactive sites for contact sensitization.³⁰ Both of these chemicals share this biophore, however, the more reactive DCC has monocyclic aliphatic ring structures as compared to the isopropyl side chains of DIC. This difference would not have been expected to confer increased reactivity to DCC since all carbon bonds for both compounds are saturated. Furthermore, the MEST was a more effective indicator of sensitization potential than the LLNA in these studies. In conducting this battery of assays for numerous years, no pattern has occurred with regards to the sensitivity of LLNA vs. the MEST. There has been a positive correlation between these assays at highly sensitizing concentrations, but at lower concentrations the sensitivity of these assays varies between chemicals. For these carbodiimides, the murine models of irritation and hypersensitivity used in this study correlate well with reported human case studies.

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