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Evaluation of the hypersensitivity potential of alternative butter flavorings

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ABSTRACT

Concern has been raised over the association of diacetyl with lung disease clinically resembling bronchiolitis obliterans in food manufacturing workers. This has resulted in the need for identification of alternative chemicals to be used in the manufacturing process. Structurally similar chemicals, 2,3-pentanedione, 2,3-hexanedione, 3,4-hexanedione and 2,3-heptanedione, used as constituents of synthetic flavoring agents have been suggested as potential alternatives for diacetyl, however, immunotoxicity data on these chemicals are limited. The present study evaluated the dermal irritation and sensitization potential of diacetyl alternatives using a murine model. None of the chemicals were identified as dermal irritants when tested at concentrations up to 50%. Similar to diacetyl (EC3 = 17.9%), concentration-dependent increases in lymphocyte proliferation were observed following exposure to all four chemicals, with calculated EC3 values of 15.4% (2,3-pentanedione), 18.2% (2,3-hexanedione), 15.5% (3,4-hexanedione) and 14.1% (2,3-heptanedione). No biologically significant elevations in local or total serum IgE were identified after exposure to 25–50% concentrations of these chemicals. These results demonstrate the potential for development of hypersensitivity responses to these proposed alternative butter flavorings and raise concern about the use of structurally similar replacement chemicals. Additionally, a contaminant with strong sensitization potential was found in varying concentrations in diacetyl obtained from different producers.

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1. Introduction

Reports of severe lung disease in employees at a microwave popcorn plant were investigated by scientists from the National Institute for Occupational Safety and Health (NIOSH) (Kanwal et al., 2006; Kreiss et al., 2002). An association was identified between the production of butter-flavored microwave popcorn and bronchiolitis obliterans (BO), a rare lung disease characterized sometimes by airway obstruction, inflammation and scarring occurring in the airways of the lung, resulting in severe shortness of breath, dry cough and sometimes death (Kanwal, 2008; Kreiss, 2007; Kullman et al., 2005; van Rooy et al., 2007). Artificial butter flavorings are proprietary mixtures which can contain more than 100 different volatile chemicals. Extensive environmental sampling initially identified the diketone diacetyl as the predominant component of the butter flavoring in plants with identified cases of BO (Boylstein et al., 2006; Martyny et al., 2008; White et al., 2010).

Published toxicity data for artificial butter flavorings and diacetyl are limited. However, several studies have used animal models to investigate the association between diacetyl exposure and BO. Hubbs et al. found that damage to intrapulmonary airways in rats inhaling butter flavoring vapors occurred after 6-h exposures to concentrations of vapors containing 285 ppm or greater of the diacetyl component (Hubbs et al., 2002). Subsequent studies indicated that pulmonary toxicity, including cellular degeneration and epithelial damage was associated with acute exposure to diacetyl (295 ppm and greater) as a single agent (Hubbs et al., 2008). Intratracheal instillation with a single dose of diacetyl (125 mg/kg) in rats also resulted in airway specific injury, increased airway resistance, lung fluid neutrophilia and extensive intraluminal airway fibrosis characteristic of BO (Palmer et al., 2011). These findings were further supported by inhalation studies in mice which demonstrated significant epithelial injury and peribronchial lymphocytic inflammation (Morgan et al., 2008). Recently, bronchiolitis obliterans-like changes were described in rats inhaling diacetyl vapors at concentrations >150 ppm for 2 weeks (Morgan et al., 2012a). Differences exist in the anatomical location of sites of

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greatest diacetyl damage in rodents and man which could be explained by species differences in sites of absorption within the respiratory tract (Morris and Hubbs, 2009; Gloede et al., 2011).

Diacetyl (also known as 2,3-butanedione, dimethyl diketone, and 2,3-diketobutane) is commonly used in the flavoring industry to add a buttery odor and flavor to food products. It is currently listed as a "high priority" chemical by the Flavor and Extract Manufacturers Association of the United States (FEMA) indicating that the chemical may pose a respiratory hazard if handled in an unsafe manner. Published toxicity studies also suggest that diacetyl and/or butter flavorings may present a health hazard. Diacetyl does not currently have an Occupational Safety and Health Association (OSHA) permissible exposure limit (PEL). However, the American Conference of Industrial Hygienists (ACGIH) has a threshold limit value (TLV; 0.01 ppm) and a short term exposure limit (STEL; 0.02 ppm) and the National Institute for Occupational Safety and Health (NIOSH) has proposed a recommended exposure limit (REL; 5 ppb) and a STEL (25 ppb) (NIOSH, 2011). Prevention measures such as substitution, engineering controls and respiratory protection aimed at lowering personal exposure levels need to be executed.

As a result of the data indicating diacetyl toxicity, many food manufacturers have turned to flavor alternatives (Barrera, 2011). However, substitutes for diacetyl may also be toxic and both OSHA and NIOSH have expressed concerns regarding their potential toxicity (OSHA, 2010; NIOSH, 2013). The toxicity of one such substitute, 2,3-pentanedione, has been investigated and the results suggest that pulmonary exposure in rats causes airway epithelia damage similar to that produced by diacetyl. Wistar-Han rats exposed to 2,3-pentanedione up to 200 ppm for 6 h/day, 5 day/week for up to 2 weeks had intraluminal and intramural fibrotic airway lesions similar to that of human BO (Morgan et al., 2012b). Studies conducted by Hubbs et al. investigating 2,3-pentanedione also found that following inhalation exposure Sprague–Dawley rats exhibited respiratory epithelial injury and respiratory toxicity comparable to that for diacetyl-induced injury (Hubbs et al., 2012). A recent analysis of butter flavorings used at a microwave popcorn plant identified the presence of 2,3-hexanedione, 2,3-heptanedione and 2,3-pentanedione in one or more of the samples using quantitative and semi-quantitative analysis (Boylstein, 2012). These alternative flavorings are being used in spite of the lack of thorough toxicological investigations (Day et al., 2011).

Our lab has previously shown that diacetyl is a chemical sensitizer when tested in the murine local lymph node assay (LLNA) (Anderson et al., 2007). In addition, reports suggest that flavoring chemicals may be associated with work-related asthma and skin diseases (Sahakian et al., 2008; Akpinar-Elci et al., 2004). This correlation suggests that in addition to BO, diacetyl and other butter flavoring chemicals may play a role in asthma and allergic disease.

The work described in this manuscript evaluates the skin sensitization potential of structurally similar alternative flavoring chemicals to begin to assess their potential role in the development of allergic disease and their safety as alternatives for diacetyl.

2. Materials and methods

2.1. Animals

Female BALB/c mice were used for the murine models. This mouse strain has a Th2 bias and is commonly used to evaluate IgE-mediated sensitization (Klink and Meade, 2003; Woolhiser et al., 2000). The mice were purchased from Taconic (Germantown, NY) at 6–8 weeks of age. Upon arrival, the animals were allowed to acclimate for a minimum of 5 days. Each shipment of animals was randomly assigned to treatment group, weighed, and individually identified via tail marking using a permanent marker or tattoo. A preliminary analysis of variance on body weights was performed to ensure a homogeneous distribution of animals across treatment groups. The animals were housed at a maximum of 5 per cage in ventilated plastic shoebox cages with hardwood chip bedding, NIH-31 modified 6% irradiated rodent diet (Harlan Teklad), and tap water was provided from water bottles, *ad libitum*. The temperature in the animal facility was maintained between 68 and 72 °F and the relative humidity between 36% and 57%. The light/dark cycle was maintained on 12-h intervals. All animal experiments were performed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited NIOSH animal facility in accordance with an animal protocol approved by the Institutional Animal Care and Use Committee.

2.2. Chemicals

The following butter flavoring chemicals were purchased from Sigma–Aldrich Chemical Company, Inc. (Milwaukee, WI) and used to generate data for Figs. 1, 2 and Table 1: 2,3-hexanedione (90%, CAS# 3848-24-6), 3,4-hexanedione (95%, CAS# 4437-51-8), 2,3-heptanedione (>97%, CAS# 96-04-8), 2,3-butanedione/diacetyl (>97%, CAS# 431-03-8), 2,3-pentanedione, (97%, CAS# 600-14-6). Diacetyl was also purchased from the producers TCI America (98%; Portland, OR), Fluka (99%; Milwaukee, WI) and Acros Organics (99%; Morris Plains, NJ) for comparison purposes to evaluate a potential contaminant (Fig. 3). Additional chemicals used for these studies were purchased from Sigma–Aldrich and include: α-hexylcinnamaldehyde (HCA, CAS 101-86-0), 2,4-dinitrofluorobenzene (DNFB, CAS 70-34-8), *O*-(2,3,4,5,6-pentafluoro-benzyl)hydroxylamine hydrochloride (PFBHA) (98+%), furfuryl propionate (>98%), 2-ethoxyethyl acetate (98%), 2-butoxyethyl acetate (99%), toluene 2,4-diisocyanate (TDI, CAS 584-84-9), 2,2,2-trifluoroethylamine hydrochloride (TFEA, 98%), N-(3-dimethylaminopropyl)–N′-ethylcarbodiimide hydrochloride (EDC ⩾ 98%), pyridine (99.8%), tert-butyl methyl ether (MTBE, ⩾99.8%). HPLC grade methanol and hexane Optima grade (95%) were purchased from Fisher Scientific (Pittsburgh, PA). Water (DI H_2O) was distilled, deionized to a resistivity of 18 MΩ cm and filtered using a Milli-Q® filter system (Billerica, MA). Helium (UHP grade), the carrier gas, and Nitrogen (UHP grade), were supplied by Butler Gas (McKees Rocks, PA) and used as received. Experiments were carried out at (297 ± 3) K and 1 atmosphere pressure. Helium (UHP grade), the GC carrier gas and nitrogen (UHP grade) were supplied by Amerigas (Sabraton, WV) and used as received.

2.3. Concentration range finding studies

Concentration range finding studies were performed to select the concentrations of the butter flavorings to be used for dermal exposures. Mice (3 per group) were exposed topically to acetone vehicle or increasing concentrations of test article (up to 50%) in acetone on the dorsal surface of each ear (25 μl per ear) for 3 consecutive days. Acetone was selected as the appropriate vehicle based on solubility, historical control data, and accepted use in skin sensitization studies (NIEHS, 1999). Animals were allowed to rest for 2 days following the last exposure and then weighed and examined for signs of overt toxicity including loss of body weight, fatigue/lack of activity, and ungroomed fur.

2.4. Combined irritancy and local lymph node assay

To determine the irritancy and sensitization potential of the butter flavorings, a combined LLNA was conducted as previously described (Anderson et al., 2007) and according to the method described in the ICCVAM Peer Review Panel report (NIEHS, 1999) with minor modifications. Briefly, mice (5 per group) were exposed topically to acetone vehicle, increasing concentrations of test agent, or positive control (HCA) on the dorsal surface of each ear (25 μl per ear) for three consecutive days. HCA (30% HCA) is an accepted and well characterized positive control for the LLNA (NIEHS, 1999). DNCB (0.3%) was used as a positive control for the irritancy portion of the experiment. For irritancy evaluation, the thickness of the right and left ear pinnae of each mouse was measured using a modified engineer's micrometer (Mitutoyo Co., Japan) before the first chemical administration and 24 h 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. Animals were allowed to rest for 2 days following the last exposure. On day 6, mice were injected intravenously via the lateral tail vein with 20 μCi ^{3}H-thymidine (Dupont NEN; specific activity 2 Ci/mmol). Five hours after ^{3}H-thymidine injection, animals were euthanized via CO_2 inhalation, and the left and right auricular draining lymph nodes (DLN; drain site of chemical application) located at the bifurcation of the jugular vein were excised and pooled for each animal. Single cell suspensions were made and incubated overnight in 5% trichloroacetic acid and samples were counted using a Packard Tri-Carb 2500TR liquid scintillation analyzer. Stimulation indices (SI) were calculated by dividing the mean disintegrations per minute (DPM) per test group by the mean DPM for the vehicle control group. EC3 values (concentration of chemical required to induce a 3-fold increase over the vehicle control) were calculated based on the equation from Basketter et al. (1999). Dosing concentrations (12.5–50%) were selected based on the results from the concentration range finding studies. The concentration of chemical required to induce a 3-fold increase over the vehicle control (EC3) was calculated based on the equations from Basketter et al. (1999).