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Allergenicity evaluation of fragrance mix and its ingredients by using *ex vivo* local lymph node assay–BrdU endpoints

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ABSTRACT

The present studies were performed to compare the differences between sensitization potency of fragrance mix and its ingredients (oak moss absolute, isoeugenol, eugenol, cinnamal, hydroxycitronellal, geraniol, cinnamic alcohol, alpha amyl cinnamal), by using *ex vivo* LLNA–BrdU ELISA. The SI and EC_3 values were calculated and potency classification was found for the mixture and for each ingredients. T_H1 cytokines (IL-2, IFN-γ) and T_H2 cytokines (IL-4, IL-5) releases from lymph node cell culture were also investigated as contact sensitization endpoints. The EC_3 values were calculated and the potency of contact sensitization were classified for fragrance mix, oak moss absolute, isoeugenol, eugenol, cinnamal, hydroxycitronellal, geraniol, cinnamic alcohol, alpha amyl cinnamal respectively: 4.4% (moderate), 3.4% (moderate), 0.88% (strong), 16.6% (weak), 1.91% (moderate), 9.77% (moderate), 13.1% (weak), 17.93% (weak), 7.74% (moderate). According to our results it should be concluded that exposure to fragrance mix does not constitute an evidently increased hazard compared to exposure to each of the eight fragrance ingredients separately. Cytokine analyses results indicate that both T_H1 and T_H2 cytokines are involved in the regulation of murine contact allergy and can be considered as useful endpoints.

1. Introduction

Allergic contact dermatitis affects approximately 1–10% of the general population (Kimber et al., 2002). The incidence of allergic contact dermatitis has a tendency to increase in the industrialized countries parallel to the increase in the number of potential sensitizers. On the other hand current population are widely exposed to fragrances because of the increased usage of the cosmetic products. Fragrance mix is an important diagnostic marker in the European baseline series based on patch test results (Johansen, 2003). It is considered as the main indicator and screening test for perfume allergy. Fragrance mix was developed at the beginning of the 1970s by Walter Larsen as a new diagnostic test for fragrance allergy. Fragrance mix has 8 ingredients, namely, 7 synthetic fragrances (cinnamal, cinnamyl alcohol, geraniol, isoeugenol, eugenol, hydroxycitronellal, alpha amyl cinnamal) and 1 natural fragrance (oak moss absolute). The initial concentration of each ingredients was 2% but this was reduced to 1% in 1984 (Larsen, 1985).

The Local Lymph Node Assay (LLNA) is an alternative to guinea pig models for the assessment of the contact sensitization potential. The contact sensitization of an allergen is characterized by lymphocyte proliferation in the lymph nodes (Basketter et al., 2007). The assay provides information on a substance's ability to induce sensitization, and importantly, delivers quantitative data that enable dose response assessment.

The use of standard LLNA is difficult in any laboratory because such radioisotope-based method requires special facilities and handling procedures. Several authors have been conducting investigations for the development of an alternative non-radioactive method for performing LLNA (Ehling et al., 2005a,b; Idehara et al., 2008; Lee et al., 2002; Takeyoshı et al., 2001, 2005, 2006; Kojima et al., 2008).

The method, the LLNA:BrdU-ELISA proposed by Takeyoshi et al. (2001) and refined during an interlaboratory validation study (Kojima et al., 2008) was approved by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2010). Although the validation of this assay was conducted, ICCVAM recommends the future studies to characterize the limitations of the LLNA:BrdU-ELISA test method (ICCVAM, 2010, 2011). By considering the limitation of this method recently we had

Abbreviations: AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care; AOO, Acetone: Olive Oil; EC_3, estimated concentration; FBS, Fetal Bovine Serum; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; IFN, interferon; IL, interleukin; LLNA, Local Lymph Node Assay; PHA-L, phytohemagglutinin-L; SI, Stimulation Index.

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developed the following *ex vivo* non-radioactive LLNA (*ex vivo* LLNA–BrdU ELISA) and we had also investigated formation/release of interleukins (IL)-2, -4, and -5, and interferon (IFN)-γ from isolated lymph node cells as contact sensitization endpoints of this method. (Ulker et al., 2011, 2013).

The current study has been conducted with three aims. First is to investigate the sensitization potency of fragrance mix by using *ex vivo* LLNA–BrdU ELISA. Second is to examine any difference in sensitization potency for the ingredients arising from their exposure in a mixture. Third is to investigate T_H1 cytokines (IL-2, IFN-γ) and T_H2 cytokines (IL-4, IL-5) releases from lymph node cell culture as contact sensitization endpoints.

2. Materials and methods

2.1. Mice

Female 8–12-week-old female Balb/c mice were obtained from Gulhane Military Medical Academy (Ankara, Turkey) and then housed at a temperature of 23 °C and relative humidity of 55% and with a 12 h light/dark cycle. Mice were provided mousechow (Optima, Kırklareli, Turkey) and water *ad libitum*. All animal procedures were conducted in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accredited facility under an animal protocol approved by the Ankara University Animal Experiments Ethics Board. Before the experiment, mice were weighed, randomized and marked for identification.

2.2. Chemicals

Fragrance mix and the ingredients were applied to the animals at different concentrations in Acetone: Olive Oil (4:1 v/v) (AOO) (Sigma). Fragrance mix was prepared by mixing eight constituents at the same volume. These constituents were alpha amyl cinnamal (RTC, USA), cinnamal (RTC, USA), cinnamic alcohol (Merk, Germany), eugenol (Acros organics, ABD), geraniol (Fluka, Germany), hydroxycitronellal (Acros organics, ABD), isoeugenol (Acros organics, ABD) and oak moss absolute (Brial, Germany).

2.3. Ex vivo BrdU incorporation

Five groups of mice ($n = 4$/group) were exposed topically on the dorsum of both ears to 25 μl of different concentration of chemicals and vehicle (AOO) alone daily for three consecutive days. The mice in this study were only rested on Day 4. On Day 5, all mice were killed by cervical dislocation and their auricular lymph nodes were excised and weighed. Excised right and left lymph nodes were pooled and homogenized, and the released cells suspended in 15 ml physiological saline. After counting, some cells from the suspension were seeded into 96-well culture plates (at 10^5 cells/well, in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum [FBS] and 1% penicillin–streptomycin (all from Bio-chrom, Berlin, Germany). After culture had occurred for 48 h at 37 °C, BrdU (10 μl of a 10 μM BrdU labeling solution; final concentration = 1 μM BrdU) was then added to the wells for a 24 h labeling period. The cells in the wells were then recovered by aspiration and the extent of BrdU incorporation measured by ELISA (Roche, Penzberg, Germany), according to manufacturer instructions. The absorbance was measured at a wavelength of 450 nm (OD_{450}) with a reference wavelength of 620 nm, using an ELISA reader (SpectraMAX, Molecular Devices Inc., Sunnyvale, CA) microplate reader; these values were used to define the BrdU labeling index.

2.4. Calculations of stimulation indices (SI)

Stimulation Index (SI) was calculated as the ratio of the mean *ex vivo* BrdU incorporation (labeling index) for each treatment group vs that of the vehicle control group.

2.5. Calculation of EC_3 value

The EC_3 – a measure of relative skin sensitizing potency of a substance – is expressed as the estimated concentration of chemical necessary to produce a 3-fold increase in proliferation in draining lymph nodes compared with the spontaneous proliferation that occurs with cells from vehicle-treated control hosts. The method represents a simple linear interpolation of the points in the dose–response curve which lie immediately above and below the classification threshold, i.e., a stimulation index of 3. If the datapoints lying immediately above and below the SI value of 3 have the co-ordinates (a, b) and (c, d), respectively, then the EC_3 value may be calculated using the following equation (Basketter et al., 2007):

$$EC_3 = c + [(3 - d)/(b - d)](a - c)]$$

2.6. Culture of lymph node cells and cytokine determinations

Harvested lymph node cells from the *ex vivo* protocols outlined above were seeded in a 24 wellculture plate (at 1×10^6 cells/well) in 1 ml of RPMI 1640 medium supplemented with 10% FBS and 1% penicillin – streptomycin. In the case of the *ex vivo* method, the cells used here were obtained from the pooled cells from Day 4-rested hosts prior to removal of aliquots for use in BrdU staining/analyses (see above). Following the seeding steps, the wells were then supplemented with 5 μg/ml of phytohemagglutinin-L (PHA-L; Biochrom). PHA-L was selected as the mitogen here (as opposed to concanavalin A) in that it has been widely used for mitotic stimulation of T-lymphocytes. After 72 h of culture in a 37 °C incubator containing 5% CO_2, supernatants were collected and stored at −80 °C until analyzed for levels of IL-2, IFN-γ, IL-4, and IL-5. The levels of each cytokine in the culture supernatants were measured using commercially available ELISA kits (BenderMed Systems, Burlingame, CA), according to the manufacturer's instructions.

2.7. Statistical analyses

Means and standard errors of mean (S.E.M.) were calculated for the cytokine levels of vehicle group and the groups exposed by topical application with the different chemicals. The differences in cytokine levels between vehicle group and each treatment group were statistically analyzed with Mann–Whitney U test and $p < 0.05$ was taken as the level of significance.

3. Results

3.1. Lymph node-related parameters and ex vivo LLNA–BrdU results

In these studies, five groups of Balb/c mice for *ex vivo* studies were exposed topically (on dorsum of both ears) to different concentration of fragrance mix, and the ingredients of fragrance mix or to AOO vehicle alone on three consecutive days (i.e., Days 1–3). In the "*Ex vivo* LLNA–BrdU ELISA" studies, mice received no BrdU injection but were rested on Day 4. One day later (i.e., 2 d after final application, Day 5), all mice were sacrificed. The auricular lymph node weights, lymph node cell counts were elevated in a manner reflecting a clear dose-related trend.

The auricular lymph node weights, lymph node cell counts, the data for all OD_{450}, SI, and EC_3 values from *ex vivo* BrdU labeling studies are also presented in Table 1. The EC_3 values were calculated and the potency of contact sensitization were classified for fragrance mix, oak moss absolute, isoeugenol, eugenol, cinnamal, hydroxycitronellal, geraniol, cinnamic alcohol, alpha amyl cinnamal respectively: 4.4% (moderate), 3.4% (moderate), 0.88% (strong), 16.6% (weak), 1.91% (moderate), 9.77% (moderate), 13.1% (weak), 17.93% (weak), 7.74% (moderate). The EC_3 results were also compared with the result of previous standard LLNA studies and were shown in Table 2.

3.2. Cytokine determinations

As described in the method part, on Day 5, auricular lymph nodes were excised from each mouse in the various treatment groups and lymph node cell suspensions were prepared. These cells were then cultured in medium in the presence of PHA-L mitogen to assess background and induci-bile formation of select T_H1 (IL-2, IFN-γ) and T_H2 cytokines (IL-4, IL-5) associated. The levels of IL-2, IFN-γ, IL-4, and IL-5 present in the lymph node cell culture supernatants are provided in Figs. 1–9. Both T_H1 and T_H2 cytokines, were increased by applied chemicals and mixtures in a dose-dependent manner with statistical significance ($p < 0.05$).

4. Discussion

The standard LLNA has several advantages since it is quicker and cheaper, providing a quantitative endpoint. However to use the assay is difficult in any laboratory because such