

Investigation of Allergenicity of Some Cosmetic Mixtures by Using ex vivo Local Lymph Node Assay-BrdU Endpoints

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Key Words

Balsam of Peru · Fragrance mix · Allergic contact dermatitis · Ex vivo local lymph node assay-BrdU · Cytokine release

Abstract

Background: Balsam of Peru and fragrance mix are commonly used in cosmetic products. Allergy to fragrance is the most common cause of cosmetic contact dermatitis. **Methods:** In the present study, ex vivo local lymph node assay-5-bromo-2'-deoxyuridine (LLNA-BrdU) was used to evaluate the dermal sensitization potential of these cosmetic mixtures. The stimulation index values and estimated concentration (EC3) values were calculated and the potency classification was found for each mixture. At the same time, in order to measure the irritant effect without having to use additional animals, a combination of ex vivo LLNA-BrdU and the irritancy assay was conducted. **Results:** Th1 [interleukin (IL)-2, interferon- γ] and Th2 cytokine (IL-4, IL-5) releases from lymph node cell culture were investigated as non-radioactive endpoints. According to the results of ex vivo LLNA-BrdU assays, EC3 values were found to be 3.09% (moderate) for balsam of Peru and 4.44% (moderate) for fragrance mix.

Cytokine analysis results indicate that both Th1 and Th2 cytokines are involved in the regulation of murine contact allergy and can be considered as useful endpoints. **Conclusion:** In conclusion, according to our results, fragrance mix and balsam of Peru can be considered as moderate sensitizers; however, in high concentrations, both of them have irritation properties. The cytokines investigated can be considered as the endpoints of the ex vivo LLNA-BrdU assay.

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Introduction

It is well known that fragrances are very important sensitizers. Fragrance mix and balsam of Peru are two important diagnostic markers in the European baseline series based on patch test results. Fragrance mix has 8 components: 7 synthetic fragrances (cinnamal, cinnamyl alcohol, geraniol, isoeugenol, eugenol, hydroxycitronellal, α -amyl cinnamal) and 1 natural fragrance (oakmoss absolute). Balsam of Peru is a natural resinous balsam with odorous properties. It consists of essential oil and resin and is thus an oleoresin. Cinnamal is a major component

of the resin [1] which also contains cinnamyl alcohol, eugenol and isoeugenol [2].

Local lymph node assay (LLNA) is an alternative to guinea pig models for the assessment of contact sensitization potential. Contact sensitization of an allergen is characterized by lymphocyte proliferation in the lymph nodes [3]. LLNA provides information on the ability of a substance to induce sensitization, and importantly, delivers quantitative data that enable dose-response assessment. Previous studies have shown that some essential oils such as basil, clove leaf and palmarosa can result in a dose-dependent local lymph node cell proliferation [4].

The original LLNA uses ^3H -methyl thymidine to measure lymphocyte proliferation. The use of this method is difficult in any laboratory because this radioisotope-based method (RI) requires special facilities and handling procedures. Several authors have conducted investigations for the development of an alternative non-RI method for performing LLNA [5–12].

The non-RI method, LLNA:5-bromo-2'-deoxyuridine (BrdU)-ELISA, proposed by Takeyoshi et al. [8], is a modification of the original LLNA. This method involves assessing lymphocyte proliferation using ELISA to measure BrdU incorporation instead of measuring the radioactivity produced by ^3H -methyl thymidine incorporation. This modification has recently been approved by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Although the validation of this assay was carried out, ICCVAM recommends future studies to characterize the limitations of the LLNA:BrdU-ELISA test method [10].

Recently, we have investigated the following non-radioactive endpoints of LLNA in response to dinitrochlorobenzene:BrdU incorporation ex vivo and in vivo, as well as the formation/release of interleukins (IL)-2, IL-4 and IL-5, and interferon (IFN)- γ from isolated lymph node cells [12]. The results of the in vivo and ex vivo assays were compatible both with one another and with standard radioactive LLNA results for dinitrochlorobenzene. Therefore, we concluded that the ex vivo BrdU incorporation method could be used as a viable alternative approach to assess the skin sensitization potential of the test compound in terms of improving animal welfare [9, 11, 12].

In the present study, a combination of ex vivo LLNA-BrdU [12] and the irritancy assay [13, 14] was conducted to evaluate the sensitization and irritation potential of fragrance mix and balsam of Peru. We also investigated Th1 (IL-2, IFN- γ) and Th2 cytokine (IL-4, IL-5) responses following the sensitization of mice.

Materials and Methods

Mice

Female BALB/c mice (8–12 weeks old) were obtained from Gulhane Military Medical Academy (Ankara, Turkey) and then housed at a temperature of 23°C and a relative humidity of 55%, with a 12-hour light/dark cycle. Mice were provided chow (Optima, Kırklareli, Turkey) and water ad libitum. All animal procedures were conducted in an 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.

Chemicals

Balsam of Peru (Fagron, Germany) at concentrations of 0, 0.5, 5, 25, and 50% and fragrance mix at concentrations of 0, 0.5, 5, 25, and 50% in acetone:olive oil (AOO, 4:1 v/v; Sigma) were applied to the animals. Fragrance mix (8%) was prepared by mixing 8 constituents in the liquid petrolatum. These constituents were α -amyl cinnamal (RTC, USA), cinnamal (RTC, USA), cinnamic alcohol (Merk, Germany), eugenol (Acros Organics, USA), geraniol (Fluka, Germany), hydroxycitronellal (Acros Organics), isoeugenol (Acros Organics) and oakmoss absolute (Brial, Germany).

Combined ex vivo LLNA-BrdU and Irritancy Assay

A combination of ex vivo LLNA-BrdU and the irritancy assay was conducted following a modification of previously published procedures [9, 13, 18]. Five groups of mice ($n = 4/\text{group}$) were exposed topically on the dorsum of both ears to 25 μl of different concentrations of mixtures and vehicle (AOO) alone, daily for 3 consecutive days. Before the first mixture application, the thickness of the right and left pinnae of each mouse ear was measured using a digital micrometer (Mitutoyo Co.) for the irritancy assay. Ear thickness measurements were taken 24 h following final exposure. The results of the irritancy assay were expressed as percent change in ear swelling using the following formula:

$$\% \text{ change in ear swelling} = [(\text{mean post-challenge ear thickness} - \text{mean pre-challenge ear thickness}) / \text{mean pre-challenge ear thickness}] \times 100.$$

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 were 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 and 1% penicillin-streptomycin; all from Biochrom). After culturing for 48 h at 37°C, BrdU (10 μl of a 10- μM BrdU labeling solution; final concentration = 1 μM BrdU) was added to the wells for a 24-hour 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 the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm (optical density, OD₄₅₀) with a reference wavelength of 620 nm, using an ELISA reader (SpectraMAX, Molecular Devices Inc.); these values were used to define the BrdU labeling index.

Table 1. Lymph node weight, cell count and % change in ear swelling values after treatment of mice with different concentrations of balsam of Peru and fragrance mix in AOO

Chemical	Vehicle	Concentration, w/v%	Lymph node weight, g	Cell count, $\times 10^6/\text{ml}$	% change in ear swelling
Balsam of Peru	AOO	–	0.016 $\pm$ 0.0018	4.90 $\pm$ 0.33	0
		0.5	0.020 $\pm$ 0.0003	5.73 $\pm$ 0.48	4.92 $\pm$ 2.88
		5	0.021 $\pm$ 0.0006**	8.00 $\pm$ 0.00*	7.00 $\pm$ 5.56
		25	0.023 $\pm$ 0.0003**	9.33 $\pm$ 1.33**	15.10 $\pm$ 2.59**
		50	0.027 $\pm$ 0.0008**	14.66 $\pm$ 1.33**	29.63 $\pm$ 2.89**
Fragrance mix	AOO	–	0.017 $\pm$ 0.0003	6.40 $\pm$ 0.22	0
		0.5	0.031 $\pm$ 0.0009	7.60 $\pm$ 0.36	5.02 $\pm$ 0.41
		5	0.038 $\pm$ 0.0002*	10.00 $\pm$ 0.57*	4.77 $\pm$ 0.40
		25	0.047 $\pm$ 0.0012**	12.03 $\pm$ 1.36**	11.90 $\pm$ 8.31*
		50	0.067 $\pm$ 0.0016**	16.00 $\pm$ 2.06**	21.04 $\pm$ 5.26**

Each value represents the mean $\pm$ SD. * $p < 0.05$, significantly different from the vehicle control (Dunnett's test). ** $p < 0.01$, significantly different from the vehicle control (Dunnett's test).

Calculations of Stimulation Indices and EC3 Value

The stimulation index (SI) was calculated as the ratio of the mean ex vivo BrdU incorporation (labeling index) for each treatment group to that of the vehicle control group.

The EC3 – a measure of relative skin-sensitizing potency of a substance – is expressed as the estimated concentration of a 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. an SI of 3. If the data points lying immediately above and below the SI value of 3 have the coordinates (a, b) and (c, d), respectively, then the EC3 value may be calculated using the following equation [15]: $EC3 = c + [(3-d)/(b-d)](a-c)$.

Culture of Lymph Node Cells and Cytokine Determinations

Harvested lymph node cells from the ex vivo protocols outlined above were seeded in a 24-well culture plate (at 1×10^6 cells/well) in 1 ml of RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. In case of the ex vivo method, the cells used here were obtained from the pooled cells from day 4 rested hosts prior to the removal of aliquots for use in BrdU staining/analyses (see above). Following the seeding steps, the wells were then supplemented with 5 $\mu\text{g}/\text{ml}$ of phytohemagglutinin-L (Biochrom). Here, phytohemagglutinin-L was selected as the mitogen (as opposed to concanavalin A) in that it has been widely used for the 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 IL-2, IFN- γ , IL-4, and IL-5 levels. The level of each cytokine in the culture supernatants was measured using commercially available ELISA kits (BenderMed Systems, Burlingame, Calif., USA), according to the manufacturer's instructions.

Statistical Analyses

Means and standard errors were calculated for the lymph node weights and for OD values obtained by ELISA for each treatment group. The difference(s) between the vehicle control and each treatment group were analyzed using Dunnett's t test.

Results

Lymph Node-Related Parameters

For the ex vivo study, five groups of BALB/c mice were exposed topically (on the dorsum of both ears) to 0.5, 5, 25, and 50% balsam of Peru, 0.5, 5, 25, and 50% fragrance mix or to AOO vehicle alone on 3 consecutive days. Auricular lymph node weights, lymph node cell counts, and the percent change in ear swelling associated with each mouse are shown in table 1. The results show that at a concentration of 5%, the application of balsam of Peru and fragrance mix in AOO caused significant increases in lymph node weights and cell counts as compared to the values in mice that received vehicle treatments only.

Ex vivo LLNA-BrdU for Balsam of Peru and Fragrance Mix

The EC3 values derived from the results of the ex vivo BrdU labeling study were calculated as 3.09 and 4.44% for balsam of Peru and fragrance mix, respectively. The data for all OD₄₅₀, SI and EC3 values from ex vivo BrdU labeling studies are presented in table 2.

Table 2. OD₄₅₀, SI and EC3 values from the ex vivo BrdU labeling studies

Chemical	Vehicle	Concentration, w/v%	OD ₄₅₀	SI	EC3, %
Balsam of Peru	AOO	–	0.125±0.006	1	3.09
		0.5	0.263±0.008	2.104	
		5	0.462±0.010	3.696	
		25	0.524±0.023	4.192	
		50	0.814±0.031	6.512	
Fragrance mix	AOO	–	0.127±0.006	1	4.44
		0.5	0.238±0.014	1.87	
		5	0.401±0.021	3.16	
		25	0.626±0.032	4.93	
		50	1.107±0.050	8.72	

The OD₄₅₀ values shown are means ± SDs.

Irritancy Assay

The results of the irritancy studies for fragrance mix and balsam of Peru are presented in table 1. A significant percentage of change in ear swelling was observed after exposure to a concentration of 25 and 50% fragrance mix and 5, 25 and 50% balsam of Peru.

Cytokine Determinations

The levels of IL-2, IFN- γ , IL-4, and IL-5 present in the lymph node cell culture supernatants are provided in figures 1 and 2.

According to the cytokine results of the group exposed to fragrance mix, it was found that the formation of IL-2, IFN- γ , IL-4, and IL-5 was strongly elevated in a manner reflecting a clear dose-related trend. The levels of IL-2, IFN- γ , IL-4, and IL-5 were significantly increased in the culture supernatants when fragrance mix was applied at concentrations of 5%.

We also observed a clear dose-related trend in the formation of cytokines of the group exposed to balsam of Peru. Significant differences in the release of cytokines were noted at a concentration of 25% balsam of Peru.

Discussion

In this study, we aimed to assess the sensitization potency of balsam of Peru and fragrance mix which are commonly used in cosmetic products by using ex vivo LLNA:BrdU-ELISA. We combined the LLNA test and the irritancy test to minimize animal use.

A concentration of 4.44% fragrance mix and a concentration of 3.09% balsam of Peru induced a 3-fold stimulation of proliferation in draining lymph nodes compared with vehicle controls. According to our EC3 values, fragrance mix and balsam of Peru can be classified as moderate sensitizers. The sensitization potency according to the EC3 values of the mixtures has been classified following the method of Gerberick et al. [15]. These mixtures are complex mixtures, and there are no LLNA data available for them. Although there is little information on the utility of LLNA to evaluate complex mixtures, some studies suggested that LLNA may be of some value in investigating the overall sensitizing capacity of a mixture [5, 16–18].

Patch testing with fragrances is an important step in the diagnosis of fragrance allergy. Different strategies have evolved for the detection of fragrance hypersensitivity. The most important one is the use of fragrance mix. Because of many false-positive irritant reactions obtained at the original concentration of fragrance mix, the final concentration was reduced from 16 to 8% (1% for each constituent) [19]. Besides the fragrance mix, balsam of Peru (10% in petrolatum) has also been used as screening substance for fragrance allergy in European standard series. Our results have shown that at high concentrations (>25%), these mixtures have irritant properties.

Changes in cytokine levels are related to T-helper cell proliferation that plays an important role in the induction and elicitation of contact sensitivity. The regulation of contact sensitivity responses by Th1 and Th2 cytokines has been studied intensively. Contact hypersensi-

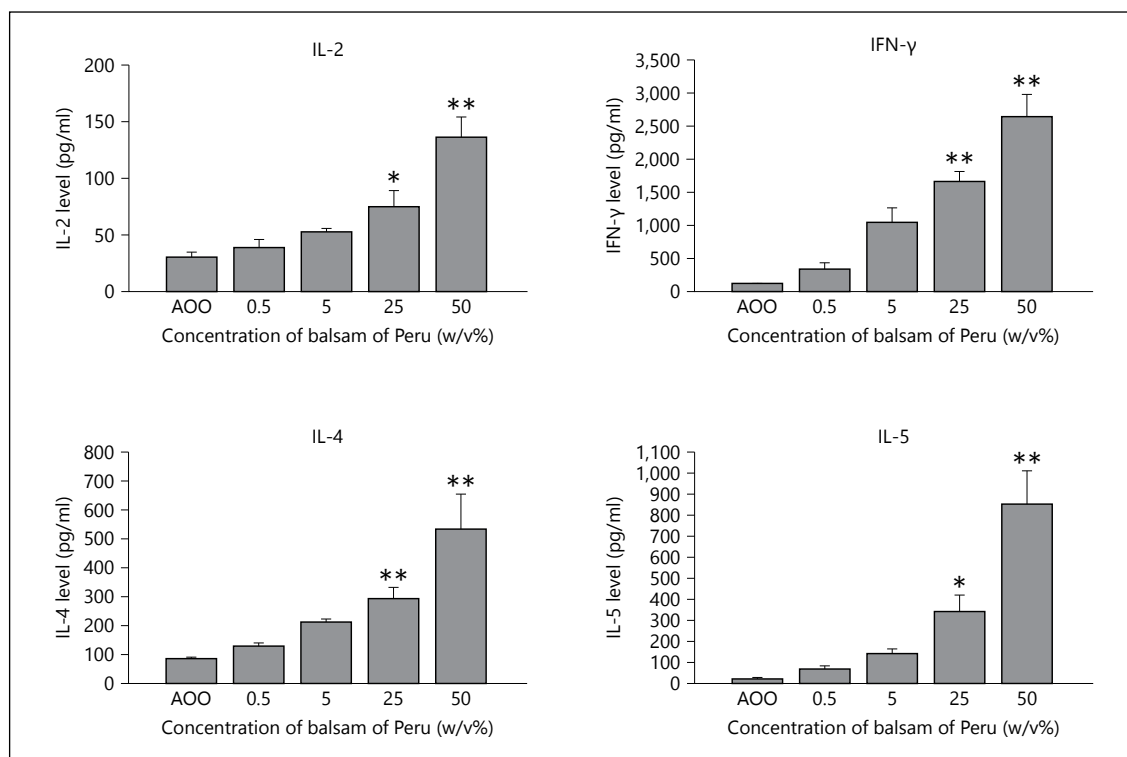


Fig. 1. The levels of IL-2, IFN- γ , IL-4, and IL-5 in the lymph node cell culture supernatants from BALB/c mice were exposed topically (on the dorsum of both ears) to 0.5, 5, 25, and 50% balsam of Peru or to AAO vehicle alone on 3 consecutive days. * $p < 0.05$, ** $p < 0.01$; significantly different from the vehicle control.

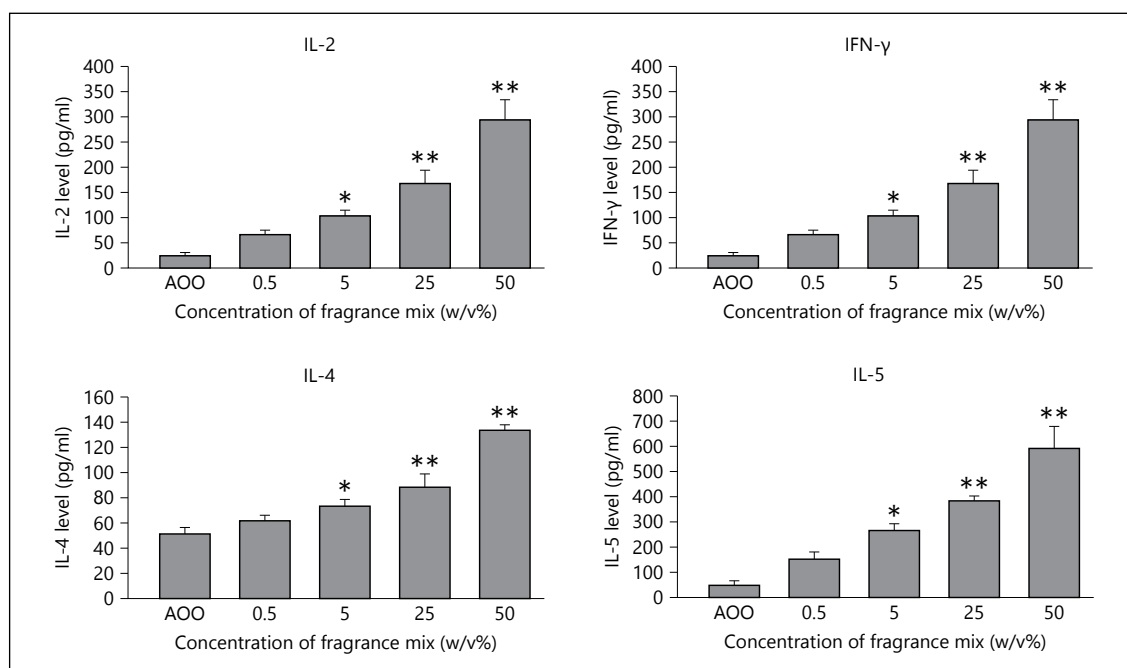


Fig. 2. The levels of IL-2, IFN- γ , IL-4, and IL-5 in the lymph node cell culture supernatants from BALB/c mice were exposed topically (on the dorsum of both ears) to 0.5, 5, 25, and 50% fragrance mix or to AAO vehicle alone on 3 consecutive days. * $p < 0.05$, ** $p < 0.01$; significantly different from the vehicle control.

tivity reactions are mainly thought to be associated with the activation of Th1 cells. In contrast, Th2 cytokines (primarily IL-4 and IL-5) have long been considered suppressive against contact sensitivity. However, in recent years, studies reported that Th2 cells and Th2 cytokines (especially IL-4 and IL-5) play a positive role in the development and pathogenesis of contact hypersensitivity [20–22]. In this study, we investigated Th1 (IL-2, IFN- γ) and Th2 cytokine (IL-4, IL-5) releases from lymph node cell culture as non-radioactive endpoints. We found that there was a dose-related increase in the formation/release of IL-2, IFN- γ , IL-4 and IL-5 for two mixtures. Our results were compatible with these latter studies.

Under the EU Cosmetics Directive (76/768/EEC), as of 11 March 2013, animal testing for cosmetic products are prohibited. Although it is prohibited to use LLNA for the testing of cosmetic ingredients because of their skin-sensitizing potential, there is still no validated in vitro method to measure the allergic potency of chemicals apart from animal testing. On the other hand, LLNA may be useful for testing chemical compounds within EU regulations such as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals). The assess-

ment of the skin sensitization potential represents an important component of the safety assessment of compounds. LLNA is the preferred testing method under REACH as it represents a reduction and refinement in animal testing (2Rs) and provides information on relative skin sensitization potency.

In conclusion, according to our results, fragrance mix and balsam of Peru can be considered as moderate sensitizers; however, in high concentrations, both of them have irritation properties. The cytokines which we investigated can be considered as the endpoints of the ex vivo LLNA-BrdU assay.

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Disclosure Statement

No conflict of interest is declared by the authors.

References

- 1 Tanaka S, Matsumoto Y, Dlova N, Ostlere LS, Goldsmith PC, Rycroft RJ, Basketter DA, White IR, Banerjee P, McFadden JP: Immediate contact reactions to fragrance mix constituents and *Myroxylon pereirae* resin. *Contact Derm* 2004;51:20–21.
- 2 Arribas MP, Soro P, Silvestre JF: Allergic contact dermatitis to fragrances: part 2. *Actas Dermosifiliogr* 2013;104:29–37.
- 3 Basketter DA, Gerberick GF, Kimber I, Lovelless SE: The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. *Food Chem Toxicol* 1996;34:985–997.
- 4 Lalko J, Api AM: Investigation of the dermal sensitization potential of various essential oils in the local lymph node assay. *Food Chem Toxicol* 2006;44:739–746.
- 5 Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, Van Loveren H, Maurer T, Riecke K, Ullmann L, Ulrich P, Vandebriel R, Vohr HW: An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: first round. *Toxicology* 2005;212:60–68.
- 6 Idehara K, Yamagishi G, Yamashita K, Ito M: Characterization and evaluation of a modified local lymph node assay using ATP content as a non-radio isotopic endpoint. *J Pharmacol Toxicol Methods* 2008;58:1–10.
- 7 Lee JK, Par JH, Park SH, Kim HS, Oh HY: A non-radioisotopic endpoint for measurement of lymph node cell proliferation in a murine allergic contact dermatitis model, using bromodeoxyuridine immunohistochemistry. *J Pharmacol Toxicol Methods* 2002;48:53–61.
- 8 Takeyoshi M, Yamasaki K, Yakabe Y, Takatsuki M, Kimber I: Development of non-radio isotopic endpoint of murine lymph node assay based on 5-bromo-2'-deoxy-uridine (BrdU) incorporation. *Toxicol Lett* 2001;119:203–208.
- 9 Ulker OC, Atak A, Ates I, Karakaya A: Evaluation of auricular lymph node cell lymphocyte proliferation and cytokine production as non-radioactive endpoints during murine contact allergy. *J Immunotoxicol* 2011;8:131–139.
- 10 Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Report March 2010. ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: BrdU-ELISA. A Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication No. 10-7552. Research Triangle Park, National Institute of Environmental Health Sciences, 2010. <http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/TMER.htm>.
- 11 Ulker OC, Atak A, Ates I, Karakaya A: Evaluation of non-radioactive endpoints of ex vivo local lymph node assay-BrdU to investigate select contact sensitizers. *J Immunotoxicol* 2013;10:1–8.
- 12 Ulker OC, Kaymak Y, Karakaya A: Allergenicity evaluation of fragrance mix and its ingredients by using ex vivo local lymph node assay-BrdU endpoints. *Food Chem Toxicol* 2014;65:162–167.
- 13 Woolhiser MR, Hayes BB, Meade BJ: A combined murine local lymph node and irritancy assay to predict sensitization and irritancy potential of chemicals. *Toxicol Methods* 1998;8:245–256.
- 14 Anderson PK, Woolhiser MR, Rase JM, Holsapple MP: Integration of primary irritation with the LLNA. *Toxicologist* 2003;72:492.
- 15 Gerberick GF, Robinson MK, Ryan CA, Dearman RJ, Kimber I, Basketter DA, Wright Z, Marks JG: Contact allergenic potency: correlation of human and local lymph node assay data. *Am J Contact Dermat* 2001;12:156–161.

- ▶16 Craig AM, Karchesy JJ, Blythe LL, Gonzales-Hernandez MP, Swan LR: Toxicity studies on western juniper oil (*Juniperus occidentalis*) and Port-Orford cedar oil (*Chamaecyparis lawsoniana*) extracts utilizing local lymph node and acute dermal irritation assays. *Toxicol Lett* 2004;154:217–224.
- ▶17 Auttachoat W, Germolec DR, Smith MJ, White KL Jr, Guo TL: Contact sensitizing potential of annatto extract and its two primary color components, cis-bixin and norbixin, in female BALB/c mice. *Food Chem Toxicol* 2011;49:2638–2644.
- ▶18 Anderson SE, Brown KK, Butterworth LF, Fedorowicz A, Jackson LG, Frasc HF, Beezhold D, Munson AE, Meade BJ: Evaluation of irritancy and sensitization potential of metal-working fluid mixtures and components. *J Immunotoxicol* 2009;6:19–29.
- ▶19 Wöhrl S, Hemmer W, Focke M, Götz M, Jarish R: The significance of fragrance mix, balsam of Peru, colophony and propolis as screening tools in the detection of fragrance allergy. *Br J Dermatol* 2001;145:268–273.
- ▶20 Itakura A, Kikuchi Y, Kouro T, Ikutani M, Takaki S, Askenase PW, Takatsu K: Interleukin 5 plays an essential role in elicitation of contact sensitivity through dual effects on eosinophils and B-1 cells. *Int Arch Allergy Immunol* 2006;140:8–16.
- ▶21 Itakura A, Ikutani M, Takatsu K, Kikuchi Y: Interleukin-5 plays a key role in mouse strain-dependent susceptibility to contact hypersensitivity through its effects on initiator B cells. *Int Arch Allergy Immunol* 2013;161:98–106.
- ▶22 Niiyama S, Tamauchi H, Amoh Y, Terashima M, Matsumura Y, Kanoh M, Habu S, Komotori J, Katsuoka K: Th2 immune response plays a critical role in the development of nickel-induced allergic contact dermatitis. *Int Arch Allergy Immunol* 2010;153:303–314.