



Increased cell proliferation in spleen and lymph nodes peripheral to contact allergen application site

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ABSTRACT

The local lymph node assay (LLNA) is widely used to identify chemicals that are contact sensitizers. The assay involves dosing mice with the chemical on both ears and pooling the superficial parotid lymph nodes for assessment of lymphocyte proliferation as a marker of sensitization. The present study explored potential reduction in animal usage by dosing one ear with the allergen and the other with vehicle-only. The respective draining lymph nodes were processed separately for tritiated thymidine (^3H -TdR) incorporation. Cell proliferation in proper axillary and renal nodes, as well as in the spleen was also assessed. Cross-contamination of the chemicals from the dosed ears to other parts of the body via preening was prevented by dosing restrained animals and washing off the residual chemical with saline after 4 h. Dosing the left ear with 0.02% oxazolone (OX) on unrestrained animals resulted in marked cell proliferation in its draining lymph node (stimulation index, SI = 12.8) and in the lymph node draining the contra-lateral vehicle-dosed ear (SI = 6), as well as the proper axillary lymph nodes (SI = 3.3). Increased ^3H -TdR incorporation was not observed in the renal lymph nodes (SI = 1.1). Similar stimulation of cells was observed in the lymph node draining the ear contra-lateral to the 30% hexylcinnamaldehyde (HCA)-dosed ear. Increased proliferative activity was observed in contra-lateral draining lymph nodes of restrained mice demonstrating that these results cannot be attributed to cross-contamination of adjacent skin. A significant increase in proliferation of splenocytes was also observed. It is concluded that dermal application of a contact allergen, as exemplified by OX and HCA, may induce cell proliferation in the neighboring lymph nodes and spleen indicative of hapten and/or haptenated proteins diffusing through the skin to peripheral nodes and the blood to produce systemic sensitization. It is also possible that lymphatic capillaries may communicate between the left and right side of the mouse head. Thus the contra-lateral draining superficial parotid node cannot be used as a control for application of contact allergen to a single ear in a modified LLNA.

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1. Introduction

The local lymph node assay (LLNA) (Kimber et al., 1994) has surpassed traditional methods such as the Guinea Pig Maximization Test (GPMT) or the Occluded Patch Test of Buehler (Buehler, 1965; Magnusson and Kligman, 1970) to become the widely accepted method for identification of the skin-sensitizing potential of chemicals. The LLNA estimates the skin-sensitizing potential of a chemical as a function of lymph node cell proliferative responses induced in mice following repeated topical exposure to the test chemical.

The formal adoption of the LLNA as a validated method after rigorous evaluations and comparisons (Dean et al., 2001; Kimber et al., 2002) has made it a stand-alone method for chemical hazard

identification and assessment. Although the LLNA has been shown to reduce assay time and animal numbers required to provide a robust assessment of chemical hazard, and to provide a reduction in dermal trauma to which animals are potentially subject, recent studies have explored ways to further reduce or eliminate the need for experimental animals in skin-sensitization safety assessments (Kimber et al., 2006; Ryan et al., 2008).

The present study examined the potential use of a modified LLNA in which the contact allergen/sensitizer is applied to one ear with the control vehicle being applied on the other ear of the mouse. This would allow reduction of animal use by elimination of separate vehicle control groups. It was hypothesized that regional lymphatic drainage is unidirectional, with lymphatics from the left and right side of the head emptying into the venous system independently (similar to human anatomy), and that chemical diffusion/absorption would be insignificant with respect to antigenic stimulation at peripheral lymph nodes and the spleen.

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2. Materials and methods

2.1. Chemicals

Oxazolone (OX), hexylcinnamic aldehyde (HCA), phosphate buffered saline (PBS), trichloroacetic acid (TCA), and acetone (A) were acquired from Sigma–Aldrich (St. Louis, MO). Tritiated thymidine (^3H -TdR, specific activity 2 Ci/mmol) was from Dupont NEN (Waltham, MA) and scintillation fluid from PerkinElmer (Waltham, MA).

2.2. Animals

Female BALB/c mice were purchased from Taconic (Hudson, NY). Animals were 6–8 weeks old upon arrival and allowed to acclimate for a minimum of 10 days. Animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility at NIOSH, Morgantown, WV. Animals were housed under controlled environmental conditions in HEPA-filtered ventilated polycarbonate cages on autoclaved hardwood Beta-chip bedding with cotton fiber nesting materials (Nestlets) and were provided Teklad 7913 food and autoclaved tap water ad libitum. All animal procedures were reviewed and approved by the NIOSH Animal Care and Use Committee.

2.3. Lymph node nomenclature

The lymph nodes examined in this study are named in accordance with the standard nomenclature proffered by Van den Broeck et al. (2006). These include the superficial parotid lymph nodes (Ln. parotidus superficialis), proper axillary lymph nodes (Ln. axillaris proprius) and renal lymph nodes (Ln. renalis). The superficial parotid lymph nodes drain the ears/head region and are the lymph nodes assayed in the LLNA. They have also been referred in the literature by various names including the cranial, cervical or auricular lymph nodes.

2.4. Experimental design

The LLNA was performed according to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) standard method (Dean et al., 2001) with modification as follows: after grouping mice into homogenous groups ($n=3-5$) based on their weight, mice were dosed with 25 μL of a test chemical (OX (0%, 0.0025%, 0.005%, and 0.02%) or HCA (0%, 7.5%, 15%, and 30%)) applied to the dorsum of the left ear of each mouse. The vehicle (25 μL acetone) was applied on the right ear of each mouse. This was repeated for 3 consecutive days. A separate control group ($n=4$) in which vehicle was dosed on both ears was run concurrently. Two hundred microliters of 0.01 M PBS containing 20 μCi ^3H -TdR was injected into the tail vein on day 6, and after 5 h the mice were euthanized via CO_2 asphyxiation. Left and right draining superficial parotid, proper axillary and renal lymph nodes were excised for each animal. The left and right superficial parotid nodes were processed separately and so were the other nodes. The spleen was also collected. For each tissue, single cell suspensions were made and following overnight incubation in 5% TCA, samples were counted using a Packard Tri-Carb 2500TR (Meriden, CT) liquid scintillation counter with subtraction of the background. Stimulation indices (SIs) were calculated by dividing the mean disintegrations per minute (DPM) per test group by the mean DPM for the vehicle control group. In one experiment both ears were dosed with 0.02% OX for comparison of proliferative activity of superficial parotid, proper axillary and renal lymph node cells.

Custom made Teflon mouse restrainers were used to examine the potential contribution of physical cross-contamination of the allergen to distal body sites through preening or contact with other animals. Animals placed in these restrainers were not able to retract their head into the restrainer or preen during the exposure period.

2.5. Statistical analysis

Evaluation of data was conducted with SigmaStat 3 from Systat Software Inc. (San Jose, CA). Data were analyzed by one-way ANOVA followed by a post hoc Bonferroni test. Comparisons between superficial parotid nodes from the same animal were done using a paired t -test. Data were considered significant at $p < 0.05$.

3. Results

OX and HCA are extreme and moderate sensitizers, respectively. Dosing mice with OX (0.0025%, 0.005%, and 0.02%) or HCA (7.5%, 15%, and 30%) on the left ear and acetone-only concurrently on the right ear resulted in dose-dependent cell stimulation in superficial parotid lymph nodes from both right and left ears (Figs. 1 and 2) compared to nodes from vehicle-only exposed mice. Quantitatively, cell proliferation in the superficial parotid lymph node contralateral to the allergen-dosed ear was always less than that observed in the superficial parotid lymph node directly draining that ear.

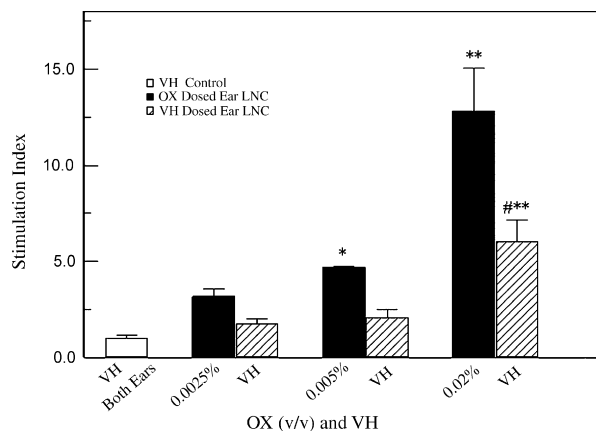


Fig. 1. Lymph node cell (LNC) proliferation measured as stimulation index (SI). Mice were dosed with OX on the left ear and the corresponding right ear was dosed with the acetone vehicle-only. A separate control group was dosed with the acetone vehicle on both ears. Dose-dependent LNC proliferation was observed in lymph nodes draining both allergen exposed and contra-lateral ears. Statistically significant stimulation ($*p < 0.05$, $**p < 0.01$) over the vehicle (both ears) control was observed with 0.005% and 0.02% OX doses. The 0.02% dose resulted in significant difference between the proliferation in the LNC draining the left ear (dosed with 0.02% OX) and the right ear that was dosed with vehicle ($\#p < 0.01$).

For example, ^3H -TdR incorporation in the contra-lateral superficial parotid lymph node cells was $46.8 \pm 9.1\%$ for 0.02% OX and $44.6 \pm 8.5\%$ for 30% HCA, of the activity in the cells from lymph nodes directly draining the allergen-dosed ears (SI = 12.8 for OX and 19.5 for HCA). The SI values for both the ipso- and contra-lateral draining nodes were determined using the ^3H -TdR incorporation of the acetone-only control group (acetone dosed on both ears). The results clearly indicate that both contact allergens (OX and HCA) induced cell proliferation in draining lymph nodes contra-lateral to the allergen-dosed ear.

Allergen induced lymph node cell proliferation was next assessed in lymph nodes caudal to the cranium. The high dose (0.02%) OX applied to both ears resulted in marked cell proliferation in the proper axillary lymph nodes (SI = 3.3 ± 1.0). Increased proliferative activity was not observed in the renal lymph nodes (SI = 1.1 ± 0.6) as exhibited in Table 1. A similar trend was observed

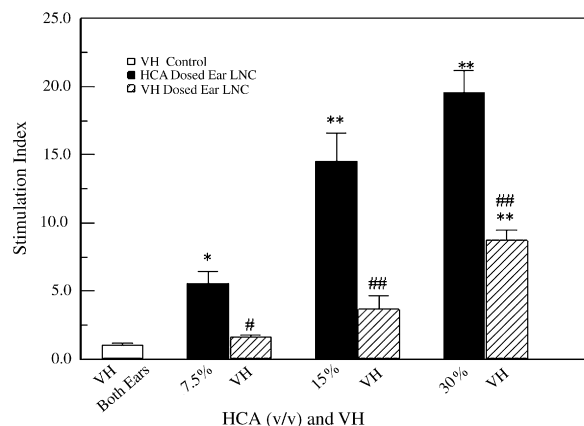


Fig. 2. LNC proliferation measured as stimulation index (SI). Mice were dosed with HCA on the left ear and the corresponding right ear was dosed with the vehicle-only. A separate control group was dosed with the acetone vehicle on both ears. Dose-dependent LNC proliferation was observed in lymph nodes draining both allergen exposed and contra-lateral ears. Statistically significant stimulation ($*p < 0.05$, $**p < 0.01$) over the vehicle (both ears acetone) control was observed with all HCA doses. All the HCA doses resulted in significant difference between the proliferation in the lymph nodes draining the left ears (dosed with HCA) and the right ears that were dosed with vehicle ($\#p < 0.05$, $##p < 0.01$).

Table 1

Oxazolone induced lymphocyte proliferation estimated as stimulation index (SI) relative to the vehicle control group which had an SI = 1. The groups with restrained animals were used.

Lymphatic organ	Allergen treatment	
	0.02% OX on left ear only SI	0.02%OX on both ears SI
Left superficial parotid lymph node	12.1 ± 3.9*	10.0 ± 2.0*
Right superficial parotid lymph node	7.6 ± 2.4*	11.3 ± 3.2*
Proper axillary lymph node	2.3 ± 0.9*	3.3 ± 1.0*
Renal lymph node	1.3 ± 0.6	1.1 ± 0.6
Spleen	2.5 ± 1.1*	3.0 ± 0.1*

* Significant increase in cell proliferation in comparison to mice dosed with acetone vehicle on both ears ($p < 0.05$).

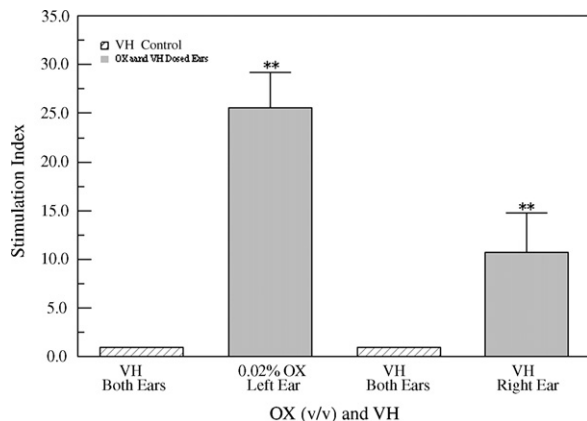


Fig. 3. Lymphocyte proliferation in the superficial parotid lymph nodes draining the 0.02% OX treated and vehicle-dosed ears of restrained animals. The corresponding vehicle controls were mice dosed with vehicle on both ears. The nodes draining the vehicle-dosed ears had lymph node cell proliferative activity 10.8 times the control indicating that restraining did not eliminate the allergen effect in the nodes draining the vehicle-dosed ears.

when only the left ear was dosed with OX and the right ear with acetone (Table 1). The proper axillary lymph node cell proliferation for the group dosed on both ears with 0.02% OX (SI = 3.3) was higher than the activity for the group dosed on one ear (SI = 2.3 ± 0.9). The group dosed on both ears with OX was exposed to twice the amount of chemical per mouse than the group dosed on one ear. The data suggest that there is some lymphatic drainage to the axillary region potentially bypassing superficial parotid lymph nodes.

The potential for physical cross-contamination within the animal from preening or contact with cage mates was investigated by dosing restrained animals (in holding tubes with only the head exposed) for 4 h and then washing off any residual chemical from the ear's surface. Fig. 3 shows the comparative cell proliferative activity in the superficial parotid nodes for the restrained animals that were dosed on one ear with 0.02% OX. A significant increase in proliferative activity in the contra-lateral lymph node which averaged 21% of the activity of the superficial parotid lymph nodes directly draining the OX dosed ears was observed.

When the spleen from the restrained animals was collected and processed for cell proliferation, a statistically significant ($p = 0.01$) increase in ^3H -TdR incorporation in splenocytes from OX-dosed mice was observed relative to the vehicle control. The splenocytes stimulation is shown in Table 1.

4. Discussion

The initial objective of the present study was to investigate the potential of modifying the LLNA to dose a single ear and use the

draining lymph node from the contra-lateral ear as a control. It was hypothesized that lymph drainage is unidirectional from the left side of the head to the thoracic duct as in humans, and allergen or activated antigen presenting cells would be greatly diluted and/or filtered through lymphatic organs distal to the thoracic duct before reaching the contra-lateral superficial parotid lymph node. The time-course of the LLNA is such that it is a measure of sensitization, not allergic contact dermatitis induction. Clearly, our hypothesis was disproven by the very high, dose-dependent proliferative cell response observed in the lymph node draining the ears contra-lateral to the allergen-dosed ears. This observation raised questions pertaining to the pattern/distribution of allergen and subsequent stimulation of peripheral lymph node and spleen cells. While anatomy of the murine lymph nodes has been well described (Van den Broeck et al., 2006), the drainage pattern and distribution of lymphatic vessels is relatively unknown.

The study was extended to include lymph nodes distant to the superficial parotid lymph nodes to the thoracic duct. Allergen induced lymph node cell proliferation was observed in the proper axillary lymph node, which receives lymphatic drainage from the forelimb. No increase in proliferative activity was observed in the renal lymph node, which is the furthest removed from the application area of the lymph nodes collected in this study. The most probable explanation of the high proliferative activity observed in the superficial parotid lymph node draining the ear contra-lateral to the allergen-dosed ear is that there is significant diffusion of chemical allergen through interstitial spaces and/or dendritic cell migration in the lymphatic drainage from the head of the mouse which crosses the midline to communicate through lymphatic vessels on the opposite side.

Increased proliferation was also observed in the spleen suggesting sufficient allergen (either as unbound hapten or protein bound) was absorbed into the blood to induce a splenic immune mediated response. After skin absorption the chemical molecules may be absorbed into either the lymphatic system or the capillaries of the blood. Blood capillaries supplying the interstitium are characterized by the presence of tight interendothelial junctions and an uninterrupted basement membrane that is only permeable to small (<1–2 kDa) moderately lipophilic molecules (Porter et al., 2001). The uptake of molecules into the blood and/or lymph is subject to the molecules passing through the extra-cellular matrix (Swartz, 2001). The evidence of allergen stimulation in the spleen after topical application is of particular interest, but an often ignored aspect of the LLNA. There is a possibility of local and systemic toxicity with attendant effects on the immune response observed with application of allergenic chemicals to the skin. For instance, local and systemic toxicity of the test chemical must be considered in the justification for using high LLNA doses ($\geq 10\%$) (Kimber et al., 2006). It must be noted, that the definition of a high dose is highly dependent on the specific chemical with respect to both allergenicity and direct local and systemic toxicity.

The potential confounders of preening or cross-contamination between animals were eliminated by preventing physical transfer of contact allergen by restraint and removal of residual surface allergen after a 4-h exposure. Allergen induced proper axillary lymph node stimulation indexes were comparable between the restrained and unrestrained groups demonstrating that cross-contamination due to preening or animal to animal contact was not responsible for the immune stimulation in sites peripheral to the allergen application area.

Even though not tested in the present study, the vehicle is known to be important in quantitative LLNA assessments. We have recently demonstrated the importance of vehicle selection in the assessment of the effect of carbon chain length on LLNA measurement of dermal sensitization by bromoalkanes (Siegel et al., 2009). The choice of a vehicle is likely to have an effect on the absorption of the

chemical into the skin and extent of diffusion sub-dermally (Jowsey et al., 2008). Acetone was chosen as the vehicle for the present studies due to (1) solubility of test chemicals and (2) its high vapor pressure. The volatility of acetone should limit its absorption into the skin and limit solvent induced allergen migration through the skin.

In conclusion, epicutaneous application of contact allergen, as exemplified by OX and HCA, induces antigen induced cell proliferation in lymph nodes that do not directly drain the dosed area, and the spleen. Thus, lymph nodes peripheral to the allergen application area in an individual animal cannot be used as controls. This study demonstrates that local dermal application of allergen can spread, most likely through absorption and diffusion potentially producing systemic sensitization.

Conflict of interest statement

No competing interests.

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