

## Alternative application route in the LLNA provides crucial environmental enrichment and broadens the usability of vehicles

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### Abstract

The Local Lymph Node Assay (LLNA), OECD Guideline 429, is the preferred animal model for assessment of skin sensitizing chemicals. A disadvantage of the OECD Guideline is that single housing of animals is required, which causes distress and discomfort to the mice.

The aim of the present work was therefore to refine housing and treatment conditions of animals in the LLNA and furthermore improve the application of water-soluble substances. Instead of applying the test material to the dorsum of the ears, we applied the test material into the ear canal. Otherwise, the standard OECD protocol with groups of CBA/Ca mice (n=5) was used.

Application into the ears has several advantages: The animals can be housed in groups; the dosing procedure can be performed by one person only; the procedure itself is gentle as the mouse does not have to be restrained. Moreover, if water is used as a vehicle it will not run off when dosed into the ears.

Conclusion: Results obtained from the application on the ear dorsum compare favorably to direct application into the ear canal. Furthermore, the alternative dosing procedure provides significant animal welfare benefits compared to the standard procedure.

**Keywords:** refinement, housing conditions, animal welfare, local lymph node assay

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### Introduction

Extensive human data show that industrial enzymes are not skin sensitizers (AMFEP, 1995). They are, however, proteins recognized as potential respiratory allergens and it is therefore a challenge to test enzymes for skin sensitizing potential. The LLNA is increasingly the preferred test to evaluate the sensitizing potential of chemicals (Jowsey, 2006; OECD No. 429, 2002). This guideline recommends certain organic solvents as vehicles and requires the animals single-housed. Enzymes are water soluble but water is not a recommended vehicle due to the difficulties in getting the solution to remain at the application site.

Further, female mice are considered social animals and individual housing reduces the welfare of the animals (Sherwin, 2002; Krohn, 2006). Therefore, refinement of the OECD LLNA was explored to overcome the problems with the aqueous solutions and the need for individual housing. Dosing into the

ear canal was assessed instead of dosing the dorsum of the ear. This allowed the animals to be group-housed and made it possible to use saline as a vehicle.

### Materials and methods

#### Animals

Young adult female CBA/CaOlaHsd mice, 8–10 weeks old, were used.

#### Chemicals

Test compounds: Protease, alpha-amylase and phytase from Novozymes A/S, Denmark. Ovalbumin, grade 5, >98% pure, Sigma-Aldrich, a non-enzyme reference protein.

Positive control: Hexyl cinnamic aldehyde (HCA) 95+%, Sigma-Aldrich.

All dosing solutions were prepared freshly for each application.

Table 1: Group means in Protease study: Vital and dead cells and incorporated  $^3\text{H}$ -Thymidine measured as disintegrations per minute (dpm) per node, converted into a stimulation index (SI)

	Cell count		Incorporated $^3\text{H}$ -Thymidin	
	Vital cells	Dead cells	dpm/node	SI
Saline 0.9%, Dorsal	$1.14 \times 10^6$	$8.91 \times 10^5$	439	
Protease 8%, Dorsal	$6.62 \times 10^6$	$1.74 \times 10^6$	4801	10.9
Saline 0.9%, in ears	$1.33 \times 10^6$	$7.81 \times 10^5$	602	
Protease 8%, in ears	$6.86 \times 10^6$	$1.70 \times 10^6$	4596	7.6
Protease 4%, in ears	$7.55 \times 10^6$	$1.60 \times 10^6$	4845	8.1
AOO, in ears	$1.58 \times 10^6$	$1.02 \times 10^6$	749	
HCA 25 %, in ears	$8.42 \times 10^6$	$1.40 \times 10^6$	6099	8.1

Table 2: Summary of LLNA responses for five substances in different vehicles in max. achievable concentrations

Test compound	Vehicle	Application route	SI	max dose
Ovalbumin, Non-enzyme reference protein	Saline	Dorsum	2.9	25 %
	Propylene glycol	Dorsum	1.5	10 %
		Ear canal	1.3	
Protease	Saline	Dorsum	10.9	8 %
		Ear canal	7.6	
	Propylene glycol	Dorsum	3.3	0.5 %
Alpha-Amylase	Saline	Dorsum	1.1	50 %
		Ear canal	2.6	
	Propylene glycol	Dorsum	1.9	25 %
	Ethanol Water	Dorsum	2.6	25 %
Phytase	Saline	Dorsum	2.1	50 %
		Ear canal	6.5	
HCA, Positive control	AOO	Dorsum	5.8	25 %
		Ear canal	6.8	

## Method

### Local lymph node assay

A standard LLNA was performed (OECD 429, Basketter 1999, Basketter 2002, Dearman 2001). Female CBA/Ca mice housed in groups ( $n=5$ ) were exposed on the dorsum or into the ear canal of both ears on days 0, 1 and 2. Dose volume was 25  $\mu\text{L}$ . The test materials were administered in various vehicles found to be most appropriate (Table 2) and at concentrations that were selected from pre-tests, maximizing the exposure but avoiding the local and systemic toxicity. The contact sensitizer, HCA, in acetone olive oil (AOO) 4:1 and AOO alone were included as positive and negative controls. On day 5, all mice were injected intravenously with 20  $\mu\text{Ci}$  of [ $^3\text{H}$ ]methyl thymidine in 250  $\mu\text{L}$  of phosphate-buffered saline (PBS). Five hours after the intravenous injection of [ $^3\text{H}$ ]thymidine, all mice were killed and the draining auricular lymph nodes were excised and weighed. Tissue from the protease treated ears was collected and examined histologically.

A single-cell suspension of lymph node cells from each animal was prepared by gentle mechanical disaggregation. Cells were washed twice and

cellularity was assessed by counting before they were precipitated in trichloroacetic acid at 4°C. Incorporation of [ $^3\text{H}$ ]thymidine was measured by  $\beta$ -scintillation counting as disintegrations per minute (dpm) per node for each animal. A stimulation index (SI) relative to the concurrent vehicle-treated control value was derived for each concentration of test material that was tested.

## Results

By using the ear canal as an alternative application route in the LLNA, it is possible to use water-based vehicles that easily run off the ear dorsum. In addition, it is possible to group-house the animals, a significant animal welfare element when using social animals like mice, as the involved test animals cannot remove the test material from each other. Further, the animals can be dosed by one person only without restraint, reducing the level of stress experienced by the animals during the dosing procedure.

The evaluation of the local lymph node cell proliferation showed that there was a statistically significant increase in both number of living cells in the single-cell suspension and the proliferation

(stimulation index) after the animals had been exposed to protease (by application to the dorsum as well as into the ear canal) and phytase (in the ear canal, only) (Table 1 & 2, Figs. 1 & 2). Lymph Node weights were significantly increased in groups treated with protease (both ways of application) and phytase (in the ear canal) compared to the control group. The lymph node weight of the positive control groups was also significantly increased compared to the respective negative control.

Among the tested enzymes, the protease showed the highest potential to stimulate the cell proliferation, while ovalbumin, used as a non-enzyme reference protein, did not produce a cell proliferation which could qualify as a contact sensitizer. Protease is more soluble in saline than in propylene glycol and it was

therefore possible to apply the protease at a maximum concentration of 8% in saline, while protease in propylene glycol had a maximum achievable concentration at 0.5%. The positive control, HCA, applied into the ear canal produced a mean SI of 8.1 which was within the historical range in the laboratory.

No significant differences in body weight gain were observed between the groups.

In the histopathological investigation of the ear tissue of mice dosed with protease, local skin irritation was noted as expected, but the changes were seen only in the vertical part of the ear canal and no differences were seen between animals dosed 8 % protease to the dorsum versus into the ear canal of both ears.

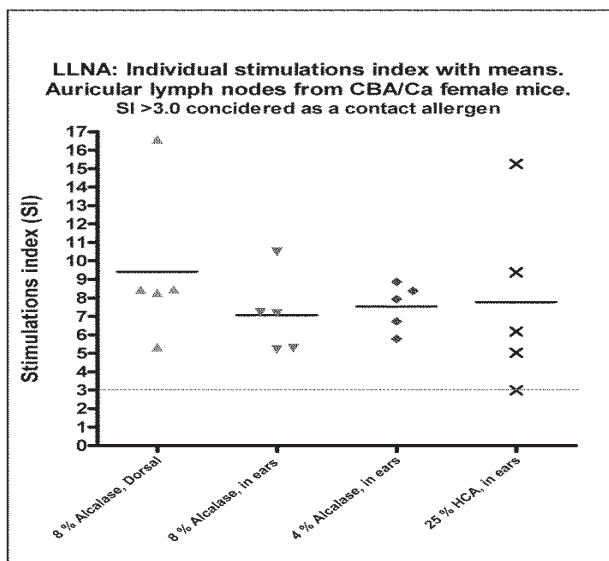


Fig. 1: Protease study: Individual & group mean stimulation index, as function of treatment groups

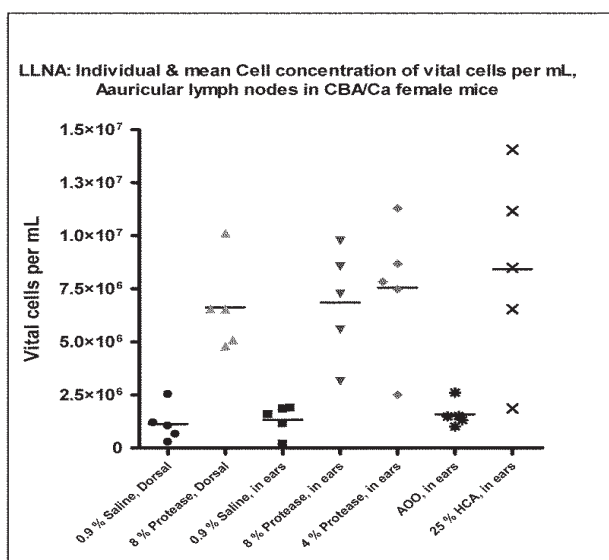


Fig. 2: Protease study, group means: Vital cell concentrations from auricular lymph nodes in CBA/OlaHSD female mice, as function of treatment groups.

## Discussion

In this work, the standard application route of the LLNA was investigated and compared with dosing into the ear canal.

When 8 % protease was applied to the dorsum of each ear lobe, the stimulation index was higher than 8 % protease applied into the ears (10.9 versus 7.6). Both application routes and concentrations resulted in positive reactions, but the application into the ears showed less group variance compared to the dorsal application. The vital mean cell concentration supports the estimation of the stimulation index, and in particular, if correct injection of <sup>3</sup>H-thymidine is questioned.

In conclusion, saline can be used as a vehicle in this modified LLNA (Table 2). The LLNA mice can be housed in groups, i.e. a clear refinement. Restraint is not needed under dosing. Body weight gain and clinical signs were either improved or not affected. HCA and AOO induced irritation near the ear drum and it is recommended to find another positive control, preferably water soluble, or use the dorsal application only for HCA in AOO.

The results indicate that enzymes are skin sensitizers. However, this is in clear conflict with experience from robust human data (AMFEP, 1995) so the present work will be continued to look into the mechanism of the reactions from these proteins, known to be potential respiratory sensitizers.

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