

Temporal Stability and Vehicle Effects on α -Hexylcinnamaldehyde Responses as a Positive Control in a Flow Cytometry-based LLNA

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#=>greater than 25% over control

1999-2005

ABSTRACT

The temporal variability and vehicle effects in the dermal sensitization response of CBA/J mice to Hexylcinnamaldehyde (HCA) administration in a Flow Cytometry-based LLNA (FC-LLNA) were analyzed. The Stimulation Index (SI) measurements over a six-year period (1999-2005) in a variety of vehicles, and the EC₃ values (that concentration of HCA calculated to yield an SI=3) were also calculated. In AOO (Acetone:Olive Oil, 4:1) vehicle, the mean SI value for 25% HCA was 9.5, and in Dimethyl sulfoxide (DMSO) and N,N-Dimethylformamide (DMF) vehicles were 9.1 and 8.4, respectively. The EC₃ value for HCA was 10.9 (\pm 4.7) for AOO vehicle, consistent with that observed in the ICCVAM validation and the literature reports for the radioactive ³H-Thymidine-based LLNA. We have also found DaAE 433 (Dimethylacetamide:Acetone:Ethanol, 4:3:3), Acetone, and Petrolatum to be useful vehicles, all producing a positive response to HCA and a range of sensitizers (SI for 25% HCA ranging from 9.1 to 18.4). The best vehicles in our lab are EtOH > Acetone > DaAE > DMSO \approx AOO \approx DMF \approx Petrolatum.

BACKGROUND

The Local Lymph Node Assay (LLNA) is a validated ICCVAM alternative dermal sensitization test developed to identify the sensitization potential of chemicals. MB Research has developed and validated the FC-LLNA, an enhanced Local Lymph Node Assay using flow cytometric endpoints and incorporating BrdU, to replace the need for radioactive isotopes. These enhancements to the standard LLNA increase sensitivity, reduce operational costs and eliminate possible hazardous radioactive exposures to humans.

The moderate skin sensitizer, HCA has been a long used positive control in the validation and conduct of the standard LLNA. In a comparison to the radioactive LLNA, we have determined that HCA responds very similarly in certain vehicles in the FC-LLNA, such as AOO.

METHODS AND MATERIALS

The ICCVAM LLNA protocol was followed in detail, with the exceptions identified below. All test materials were applied topically once daily for 3 days to the dorsum of each ear of female CBA/J mice (8-12 weeks old; n = 5). Three days after the last treatment, the mice were injected with the thymidine analog BrdU, which then becomes incorporated into proliferating lymph node cells. Five hours after injection, the mice were euthanized and the auricular nodes were collected and pooled on an individual animal basis. Lymph nodes were processed into single-cell suspensions in a microtube with a disposable pestle in PBS. The cells were centrifuged, washed, and re-suspended in cold PBS and refrigerated overnight.

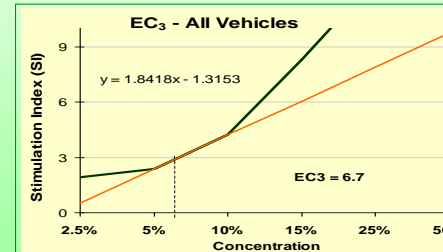
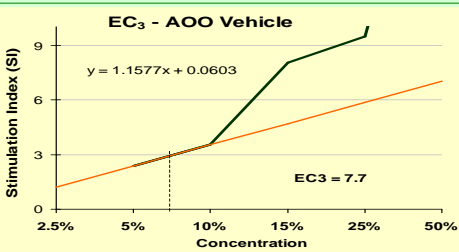
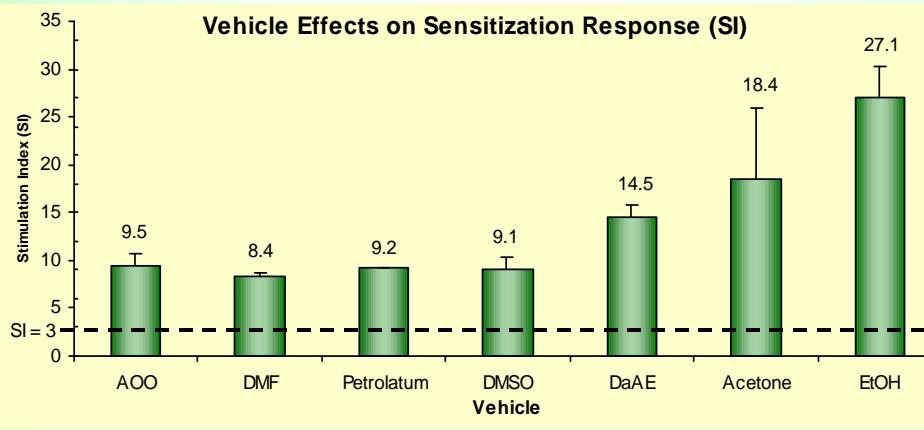
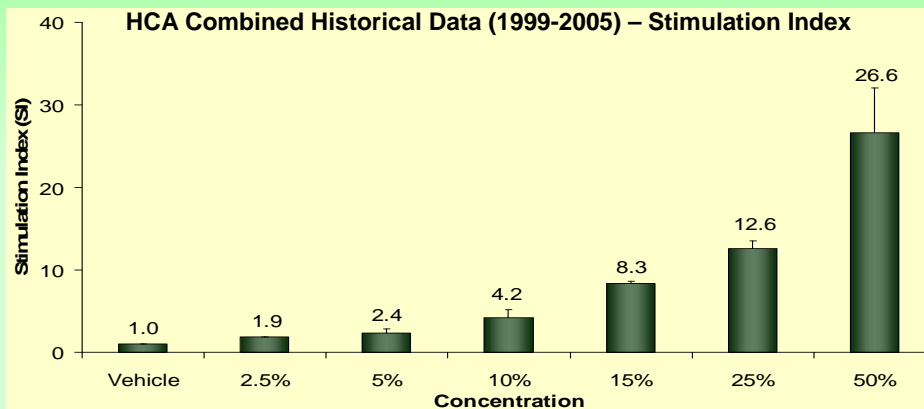
Cell Proliferation (total LNC number): Aliquots of LNC were fixed by re-suspending in 70% EtOH. Fixed cells were acid and TX-100 permeabilized, neutralized and stained with FITC-conjugated anti-BrdU antibody for flow cytometric analysis. The % BrdU-positive was determined and number of proliferating (BrdU+) cells was calculated. The number of BrdU+ LNC is calculated using the following equation:

$$\text{Total \# cells per node set} \times [\% \text{ BrdU+}] = \# \text{ BrdU+ cells/mouse}$$

The stimulation index (SI) of a compound is the ratio of the mean # of proliferating [BrdU+] lymph node cells in test article-treated groups relative to # of BrdU+ LNC in vehicle-treated groups. Test articles that yield a SI \geq 3 are characterized as sensitizing substances.

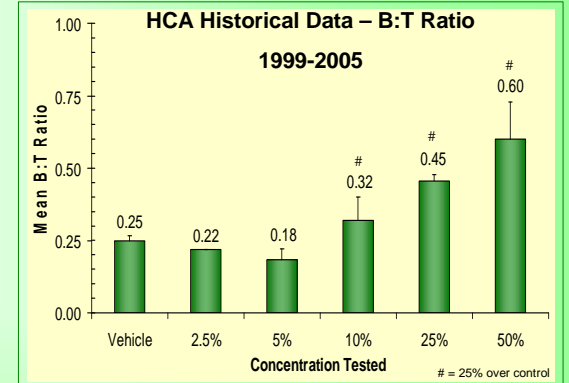
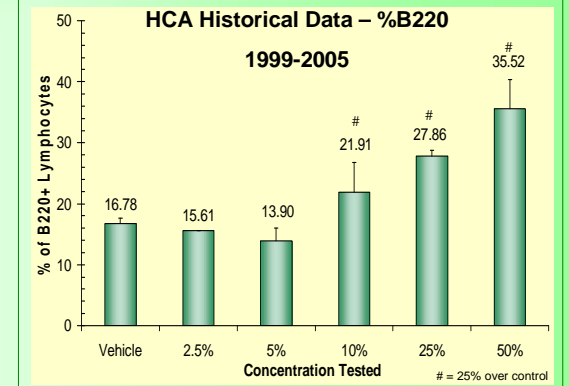
$$\frac{\# \text{ BrdU+ LNC from treated group}}{\# \text{ BrdU+ LNC from vehicle group}} = \text{SI}$$

RESULTS



METHODS AND MATERIALS (CONT)

For each group, the mean # of BrdU+ LNC was calculated and divided by the vehicle control group to obtain the SI for each individual animal. Flow cytometry was conducted using a GLP-validated, enhanced FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an Omnichrome 25 mW argon laser emitting at 488 nm with 15 mW of power and an Automated Microsampling System (Cytek Development, Fremont, CA). The histograms generated in these experiments were analyzed using WinFCM Software (Applied Cytometry Systems, Sheffield, UK) and CellQuest Flow Cytometry Software (Becton Dickinson, San Jose, CA).



CONCLUSIONS

Over the 6 year development and validation period of the Flow Cytometry-based LLNA, we have demonstrated the temporal stability of HCA as an effective positive control and the effectiveness of the Flow cytometry-based Local Lymph Node Assay.

We have also demonstrated that the SI of HCA is effected by different vehicles by examining a 25% concentration of HCA in AOO, DMF, Petrolatum, DaAE, Acetone and EtOH.

The project described was supported by grant number R44-ES10234-02 from the National Institute of Environmental Health Sciences (NIEHS), NIH. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH.

Special thanks to BD Pharmingen, San Jose, CA for collaboration on this and other research projects at MB Research Laboratories aimed expanding the use of flow cytometry in alternative toxicology methods.