TRADE SECRET

Study Title

H-24335: Local Lymph Node Assay (LLNA)

Laboratory Project ID: DuPont-3823

AUTHOR: Gregory S. Ladics, Ph.D., D.A.B.T.

STUDY COMPLETED ON: April 28, 2000

PERFORMING LABORATORY: E. I. du Pont de Nemours and Company
Haskell Laboratory for Toxicology and Industrial Medicine
Elkton Road, P.O. Box 50
Newark, Delaware 19714-0050

WORK REQUEST NUMBER: [redacted]

SERVICE CODE NUMBER: [redacted]

CERTIFICATION

I, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Issued by Study Director: Gregory S. Ladics 28-April-00

Gregory S. Ladics, Ph.D., D.A.B.T.
Senior Research Scientist

Date

TABLE OF CONTENTS

LIST OF TABLES

STUDY INFORMATION

Substance Tested: [redacted]

Synonyms/Codes: • H-24335
[redacted]

Haskell Number: 24335

Composition: [redacted]

Known Impurities: [redacted]

Physical Characteristics: [redacted]

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Sponsor: E. I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

Study Initiated/Completed: January 27, 2000 / (see report cover page)

In-Life Initiated/Completed: April 1, 2000 / April 7, 2000

STUDY PERSONNEL

The following individuals participated in the conduct of this study:

Study Director: Gregory S. Ladics, Ph.D., D.A.B.T.

Management: Judith C. Stadler, Ph.D., D.A.B.T.

Primary Technician: Charlene Smith, S.A.

Toxicology Report Preparation: Brenda Tiffin

Laboratory Veterinarian: Wanda L. West, D.V.M., A.C.L.A.M.

SUMMARY

The objective of this study was to evaluate the potential of H-24335 to produce a dermal sensitization response in mice using the local lymph node assay (LLNA). Six groups of female CBA/JHsd mice were dosed for 3 days with 0 (vehicle), 5%, 10%, 25%, 50%, or 100% H-24335 on the dorsal side of both ears. Dimethylsulfoxide was used as the diluting vehicle. A seventh group of 6 female mice was dosed for 3 days with 25% Hexylcinnamaldehyde (HCA) as a positive control. On test day 5 of the assay, mice received ^{3}-H-Thymidine by tail vein injection and were sacrificed 5 hours later. The cell proliferation in the draining auricular lymph nodes of the ears was then evaluated and compared to control.

There were no clinical signs of toxicity observed in the study. There were no statistically significant differences in mean body weights and mean body weight gains between the vehicle control group and any test substance group.

No statistically significant differences were seen in cell proliferation measurements at any test concentration. Stimulation indexes were less than 3.0 at all concentrations.

A 25% concentration of the positive control, HCA, produced a positive dermal sensitization response in mice. Therefore, the LLNA test system was valid for this study with H-24335. Under the conditions of this study, H-24335 did not produce a positive dermal sensitization response in mice.

INTRODUCTION

The purpose of this study was to examine the dermal sensitization potential of H-24335 using the local lymph node assay (LLNA). Following the topical application of the test article to the dorsal side of both ears, the dermal sensitization potential of the test article was evaluated by measuring the proliferation of lymphocytes (via radiolabel uptake) obtained from the auricular lymph nodes (i.e., the lymph nodes that drain the ears). Because H-24335 is a [redacted] and did not appear to have severe skin irritating capability (pH ~4-5), the 100% concentration was used as the high dose. For subsequent dilutions, the test article was found to be soluble in dimethlysulfoxide (DMSO).

MATERIALS AND METHODS

A. Test Species

The Local Lymph Node Assay with H-24335 was conducted in female CBA/JHsd mice.

On March 16, 2000, 48 female mice, with an assigned birth date of January 28, 2000, were received from Harlan Sprague Dawley, Fredrick, Maryland.

The CBA/JHsd mouse was selected to conduct the LLNA because Haskell Laboratory has an extensive database with this strain. Furthermore, the strain has undergone extensive interlaboratory validation with the LLNA.[1-3]

B. Body Weight Measurements

All mice were weighed on test day 0 and prior to sacrifice on test day 5.

C. Test Substance

The test substance, H-24335, was supplied by the sponsor as a [redacted] The test substance was vortex to mix before each amount for dosing was removed. The test substance appeared to be stable under the conditions of the study. No evidence of instability, such as a change in color or physical state, was observed.

D. Clinical Observations

Cage-site examinations to detect moribund or dead mice and abnormal behavior and appearance among mice were conducted at least once daily throughout the study. At every weighing, each mouse was individually handled and examined for abnormal behavior and appearance.

E. Local Lymph Node Assay

The test substance was prepared as a solution in DMSO except the 100% concentration. Twenty-five µl of H-24335 was administered topically to the dorsum of each ear of mice (6 per group) for 3 consecutive days (test days 0-2) at dosages of 0% (DMSO vehicle), 5%, 10%, 25%, 50%, and 100%. One group of 6 female mice was dosed with 25% hexylcinnamaldehyde (HCA) as a positive control. Test days 3-4 were days of rest followed by intravenous injection of 20µCi of ^{3}H-Thymidine per mouse on the morning of test day 5. One mouse in the 10% concentration group was not properly injected with the appropriate amount of ^{3}H-Thymidine and was excluded from study. Approximately 5 hours later, animals were sacrificed, draining auricular lymph nodes were removed, and single cell suspensions were prepared. The single cell suspensions were then incubated at 2-8°C overnight. Disintegrations per minute (dpm) data were obtained on test day 6 by counting the single cell suspensions on a beta counter.

A stimulation index (SI) was derived for each experimental group by dividing the mean dpm of the experimental group by the mean dpm of the vehicle control group. Statistical significance in the test article groups compared to control and/or SIs of greater than or equal to 3.0 indicated a positive response.

F. Statistical Analyses

Significance was judged at $p < 0.05$ except for dpm data which was judged at $p < 0.01$.

		Method of Statistical Analysis	
Parameter	Preliminary Test[a]	If preliminary test is not significant	If preliminary test is significant
Body Weight Body Weight Gain Lymph Node DPM Data[b,c]	Levene's test[(4)] for homogeneity and Shapiro-Wilk test[(5)] for normality[d]	One-way Analysis of Variance;[(6)] followed, with Dunnett's test[(7)]	Kruskal-Wallis test;[(8)] followed, with Dunn's test[(9)]
		and/or Sequential application[(10)] of the Jonckheere-Terpstra trend test[(11)]	

a Preliminary test not required when running only the Jonckheere-Terpstra trend test.
b If more than 1 mouse was eliminated from a group, statistics were not done on the data for that group.
c Positive control dpm data was not included in the statistical analysis.
d If the Shapiro-Wilk test was not significant but Levene's test was significant, a robust version of Dunnett's test was used.

RESULTS AND DISCUSSION

A. Clinical Signs of Toxicity, Body Weights, and Body Weight Gains
(Tables 1-2)

There were no clinical signs of toxicity observed in the study. There were no statistically significant differences in mean body weights and mean body weight gains between the vehicle control group and any test substance group.

B. Stimulation Index Data
(Table 3)

No statistically significant differences were seen in cell proliferation measurements at any test concentration. Stimulation indexes were less than 3.0 at all concentrations of H-24335.

CONCLUSIONS

A 25% concentration of HCA produced a positive dermal response in mice. Therefore, the LLNA test system was valid for this study with H-24335. Under the conditions of this study, H-24335 did not produce a positive dermal sensitization response in mice.

RECORDS AND SAMPLE STORAGE

The raw data, and the final report will be retained at Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware.

REFERENCES

1. Loveless, S.E., Ladics, G.S., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Scholes, E.W., House, R.V., Hilton, J., Dearman, R.J., and Kimber, I. (1996). Further Evaluation of the Local Lymph Node Assay in the Final Phase of an International Collaborative Trial. *Toxicology* **108**, 141-152.

2. Kimber, I., Hilton, J., Dearman, R.J., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Scholes, E.W., Ladics, G.S., Loveless, S.E., House, R.V., and Guy, A. (1995). An International Evaluation of the Murine Local Lymph Node Assay and Comparison of Modified Procudures. *Toxicology* **103**, 63-73.

3. Kimber, I., Hilton, J., Dearman, RJ., Gerberick, G.F., Ryan, C.A., Basketter, D.A., Lea, L., House, R.V., Ladics, G.S., Loveless, S.E., and Hastings, K.L. (1998). Assessment of The Skin Sensitization Potential of Topical Medicaments Using The Local Lymph Node Assay: An Inter-Laboratory Exercise. *J. Toxicol. Environ. Health*, **Part A 53(7)**, 563-579.

4. Levene, H. (1960). Robust test for equality of variances. In *Contributions to Probability and Statistics* (I. Olkin, ed.), pp 278-292. Stanford University Press, Palo Alto, CA.

5. Shapiro, S.S. and M.B. Wilk, (1965). An analysis of variance test for normality (complete samples). *Biometrika* **52**, 591-611.

6. Snedecor, G. W. and W. G. Cochran, (1967). *Statistical Methods*, 6th ed., pp. 246-248, 349-352. The Iowa State University Press, Iowa.

7. Dunnett, C.W., (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Amer. Statist. Assoc.* **50**, 1096-1121.

8. Kruskal, W.H. and W.A. Wallis, (1952). Use of ranks in one-criterion analysis of variance. *J. Amer. Statist. Assoc.* **47**, 583-621.

9. Dunn, O.J., (1964). Multiple contrasts using rank sums. *Technometrics* **6**, 241-252.

10. Selwyn, M. R., (1995). The Use of Trend Tests to Determine a No-Observable-Effect Level in Animal Safety Studies. *Journal of the American College of Toxicology*, **14(2)**, 158-168.

11. Jonckheere, A. R., (1954). "A Distribution-Free K-Sample Test Against Ordered Alternatives," *Biometrika* **41**, 133-145.

TABLES

TABLE 1

MEAN BODY WEIGHTS(g)

GROUP:	II	IV	VI	VIII	X	XII	XIV
CONCENTRATION (%): DAYS ON TEST	0	5	10	25	50	100	25*
0	20.7 (1.4)	20.8 (1.3)	20.6 (1.0)	20.4 (1.0)	20.7 (0.9)	20.7 (0.9)	20.4 (1.1)
5	21.3 (1.2)	21.4 (0.8)	21.4 (0.7)	21.2 (1.4)	21.2 (0.7)	21.3 (1.0)	21.1 (1.4)

Standard deviation is reported in parenthesis.
Statistical method: Jonckheere-Terpstra trend test was performed on data.
There were no statistically significant differences at $p > 0.05$
* HCA (positive control).

TABLE 2

MEAN BODY WEIGHT GAINS(g)

GROUP:	II	IV	VI	VIII	X	XII	XIV
CONCENTRATION (%): DAYS ON TEST	0	5	10	25	50	100	25*
0-5	0.6 (0.4)	0.6 (0.8)	0.8 (0.5)	0.8 (0.7)	0.5 (0.4)	0.5 (0.4)	0.7 (0.9)

Standard deviation is reported in parenthesis.
Statistical method: Jonckheere-Terpstra trend test was performed on data.
There were no statistically significant differences at $p > 0.05$
* HCA (positive control).

TABLE 3

STIMULATION INDEX (SI) DATA

GROUP	MATERIAL TESTED	n	SI
II	0% (DMSO)	6	N/A
IV	5% H-24335	6	0.78
VI	10% H-24335	5	1.15
VIII	25% H-24335	6	1.15
X	50% H-24335	6	1.37
XII	100% H-24335	6	1.5
XIV	25% HCA in DMSO (positive control)	6	3.98*

N/A = Not applicable
n = Number of animals per group. There were no statistically significant differences at $p > 0.01$ by Jonckheere-Terpstra trend and Dunnett's Tests.
* = DPM Positive control data was not included in the statistical analysis.