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AR226-3831

MB Research Laboratories

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VOLUME I

Study Title	:	Local Lymph Node Assay in Mice (LLNA)
Test Article	:	MTDID 6675, I
Positive Control	:	alpha-hexylcinnamaldehyde (HCA) Technical Grade 85%, Aldrich Lot/Batch #13102MO
Vehicle	:	Dimethyl Sulfoxide (DMSO), Sigma-Aldrich Batch/Lot #04734ME
Author	:	Melissa Kirk, Ph.D., Study Director
Study Completed On	:	October 25, 2007
Performing Laboratory	:	MB Research Laboratories 1765 Wentz Road P.O. Box 178 Spinnerstown, PA 18968
MB Research Project #	:	MB 07-15961.26
MB Research Protocol #	:	5650M
Sponsor	:	3M Corporate Toxicology & Regulatory Services 3M Center, Bldg 0220-06-E-03 St. Paul, MN 55144-1000
Sponsor Study#	:	07-106
Citation	:	Melissa Kirk, Ph.D. (2007) Unpublished Report by MB Research Laboratories

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Study Title	:	**LLNA**
Project #	:	**MB 07-15961.26**
Protocol	:	**5650M**

GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

This study was conducted in accordance with the Good Laboratory Practice requirements of EPA, 40 CFR 160 and 792, FDA 21 CFR 58, and as specified in Principles on Good Laboratory Practices, published by the Organization for Economic Cooperation & Development (OECD), 1997, with the following exceptions:

Test article characterization was provided but did not include stability. In addition, the test article characterization was not performed under the Good Laboratory Practices.

STUDY DIRECTOR :

Melissa Kirk, Ph.D — Date
MB RESEARCH LABORATORIES

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PROJECT NUMBER : MB 07-15961.26
TEST ARTICLE : MTDID 6675,
SPONSOR : 3M CORPORATE TOXICOLOGY & REGULATORY SERVICES
TITLE : Local Lymph Node Assay in Mice (LLNA)
PROTOCOL # : 5650M

ABSTRACT

Method Synopsis: A preliminary dermal irritation screen was conducted with three groups of healthy female CBA/J mice to determine the concentrations of the test article, MTDID 6675, , to be used in the definitive Local Lymph Node study. Three concentrations (25%, 50% and 100%) of the test article were tested (2 animals per group). These concentrations corresponded to approximately 7.5%, 15% and 29.9% of the active ingredient in the test article. This preliminary screen showed no irritation at any of the concentrations tested including the maximum soluble concentration of 100%. Based on these results, concentrations of 25%, 50% and 100% of the test article were chosen for the definitive Local Lymph Node study by the Study Director in consultation with the sponsor.

For the definitive study, three separate groups of healthy female CBA/J mice (5 animals per group) were treated with increasing concentrations of MTDID 6675, vehicle control group of five mice was treated with DMSO and another group of five mice was treated with the positive control, 25% HCA (in DMSO), in the exact same manner. The naive control group was left untreated to provide baseline values and to provide comparison for the 100% test article dose group, which did not use DMSO as a vehicle. The test article solutions, vehicle control, and positive control were administered by topical application to the dorsum of each ear, once daily for three consecutive days.

The mice were given an intraperitoneal injection of the thymidine analog 5-bromo-2'-deoxy-uridine (BrdU) five days following the initial dose, and five hours prior to sacrifice. At sacrifice, the auricular lymph nodes were isolated, single-cell suspensions of lymph node cells (LNC) were generated, and the LNC suspension was analyzed by flow cytometry for BrdU incorporation and the total number of LNC. The amount of proliferating (#BrdU+) LNC was determined as a measure of the proliferative response of the local lymph node. The stimulation index (SI) was calculated by dividing the proliferative response (BrdU incorporation) of each test article group by the proliferative response of the vehicle control group, or, for the 100% test article group, by the proliferative response of the naive control group. Test articles that yielded a SI ≥ 3 were characterized as potentially sensitizing substances.

Aliquots of the LNC suspension were stained with antibodies for immunophenotyping (B and T cells) evaluation. Cells were washed and resuspended in Phosphate Buffered Saline (PBS) containing the viability dye 7AAD. Live cells were analyzed by flow cytometry to determine %B cells, %T cells, and the B:T cell ratio. Test articles inducing an increase of ≥25% (1.25-fold) over the vehicle (or naive) control in % B cells or the B:T ratio were considered sensitizing substances.

Summary: All animals survived the in-life phase of the study and appeared normal, except for one animal in the 100% dose group, which appeared emaciated and exhibited a wet anogenital area on Day 6 of the definitive study. This animal could not be injected with BrdU, so lymph nodes were not harvested. The signs observed in this animal are not believed to be test article-related since all other animals in this group appeared normal. There were no difficulties in administration of the test article or with its adherence to the dosed ears. Body weight changes were normal, except for the animal in the 100% dose group, which

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ABSTRACT (cont'd)

Summary (cont'd):

lost 1.4 grams and appeared emaciated. Ear swelling measurements and individual animal observations indicated that none of the test article treatments resulted in dermal irritation. The SI of the positive control, 25% HCA, was 15.3, consistent with historical data. The SI values for test article MTDID 6675, at 25%, 50% and 100% were 0.8, 2.4 and 3.8, respectively. The SI value of 100% MTDID 6675 compared to the Naïve control group was 8.9.

The test article elicited a fold increase in %B cells of 1.05 at 25%, 1.22 at 50% and 1.91 at 100%, compared to the vehicle control, and an increase of 1.62-fold at 100% compared to the naïve control. Fold increases in B:T ratio were 1.04 at 25%, 1.20 at 50%, and 2.21 at 100%, compared to vehicle, and 1.79 at 100% compared to the Naïve control.

Conclusion: Topical application of the test article MTDID 6675, at 100% (29.9% as the active ingredient) resulted in a stimulation index greater than 3 (SI > 3), as well as a >25% increase in %B cells and in the B:T ratio compared to both vehicle and naïve controls. Therefore, this test article is a dermal sensitizer in the Local Lymph Node Assay. The test article has a calculated EC3 value of 54.8% (16.4% as the active ingredient). Based on ECETOC criteria, the active ingredient in MTDID 6675, can be classified as a weak sensitizer.

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Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

OBJECTIVE

To determine the sensitizing potential of topically applied test substances. This study is designed to be a stand-alone alternative assay for the Buehler Guinea Pig Sensitization Assay defined in the ICCVAM report (Federal Register 63 CFR 37405-6, July 10, 1998), EPA Health Effects Test Guideline, OPPTS 870.2600 final guideline effective March 2003, and OECD Test Guideline 429, effective April 2002.

TEST ARTICLE

Identity	:	MTDID 6675,
Test Article Characterization	:	See Appendix K for Test Article Characterization and Certificate of Analysis.
Provided by	:	3M Corporate Toxicology & Regulatory Services
Stability	:	Not supplied by the sponsor.
Date Received	:	07/06/07
Storage	:	Room temperature and humidity
Description	:	Clear liquid
Sample Preparation	:	The test article was supplied as an aqueous solution containing 29.9% of the active ingredient. It was used as received for the 100% concentration, and diluted with the vehicle, DMSO, to appropriate concentrations.

POSITIVE CONTROL

Identity	:	alpha-hexylcinnamaldehyde (HCA) Technical Grade 85%, Aldrich Lot/Batch #13102MO
Provided by	:	MB Research Laboratories
Date Received	:	05/09/06
Expiration Date	:	11/09/07
Storage	:	Room temperature and humidity
Description	:	Yellow liquid
Sample Preparation	:	294 µl of 85% HCA was added to 706 µl of DMSO, to yield a 25% solution.

VEHICLE

Identity	:	Dimethyl Sulfoxide (DMSO), Sigma-Aldrich Batch/Lot #04734ME
Provided By	:	MB Research Laboratories
Received Date	:	04/30/07
Expiration Date	:	04/30/08
Storage	:	Room temperature and humidity
Description	:	Clear liquid
Sample Preparation	:	Used as received.

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MB Research Laboratories

Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

TEST DATES

Study Initiation	(date protocol signed)	:	07/18/07
Experimental Start Date	(1st exposure to test substance)	:	07/18/07
Experimental Term Date	(last date data collected)	:	08/02/07
Draft Report Signed	(if applicable)	:	09/12/07
2nd Draft Report Submitted	(if applicable)	:	10/04/07
Final Report Signed	(study completion)	:	10/25/07

EXPERIMENTAL DESIGN

Test Animals:

Female CBA/J mice were received from Jackson Laboratories on 06/26/07, 07/10/07 & 07/17/07[1]. Following an equilibration period of at least five days, thirty-six nulliparous, non-pregnant female mice were assigned to the applicable groups using a computer-based random number program.

The animals were born on 05/01/07, 05/15/07 & 05/22/07, ±3 days. The Day 1 body weight range for the definitive study animals was 16.7 - 22.2 grams. The Day 1 body weight range for the screen animals was 19.3 - 22.0 grams. The weight variation of the animals at study initiation did not exceed ± 20% of the mean body weight.

The animals were identified by cage notation and indelible tail marks, and housed 1/cage in suspended wire cages. Bedding was placed beneath the cages and changed at least three times/week. Fresh PMI (Diet #5001) and water were freely available at all times. The animal room, reserved exclusively for mice on acute tests, was temperature-controlled, had a 12-hour light/dark cycle, and was kept clean and vermin free.

[1] *See Appendix J for Animal Health Report*

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EXPERIMENTAL DESIGN (cont'd)

Dosing:

Irritation Screening Study: Three groups of CBA/J mice (2 animals per group) were treated with three concentrations (25%, 50% and 100%) of MTDID 6675, in DMSO, for three consecutive days. These concentrations corresponded to approximately 7.5%, 15% and 29.9% of the active ingredient in the test article. Observations for edema and/or erythema and measurements of ear thickness were performed on Day 1 prior to dosing, on Day 3 before the third test article application (approximately 48 hr after the first test article application), and on Day 6 before sacrifice (approximately 120 hr after the first dose and 72 hr after the third dose). On Day 6, the mice were sacrificed using CO_2 asphyxiation. Changes in ear thickness on Day 3 and Day 6 relative to Day 1 were expressed as a percent of the Day 1 pre-dose values. Ear thickness changes of more than 25% were considered biologically significant (based on historical laboratory data) and indicative of a primary dermal irritation response.

Definitive Study: The test article concentrations used in the definitive LLNA study were chosen such that the maximum concentration tested was the highest achievable solution of the test article as supplied by the sponsor.

Six groups of CBA/J mice (5 animals per group) were treated by topical application of the test article concentrations, vehicle or positive control to the dorsum of each ear once daily for three consecutive days (at approximately the same time each day). Concentrations of 25%, 50% and 100% of the test article, corresponding to approximately 7.5%, 15% and 29.9% of the active ingredient, were tested. The test article concentration, positive control or vehicle was spread over the entire dorsal surface of the ear using a micropipette at 25 µl/ear. A naïve group of 5 animals was left untreated. The default vehicle, Acetone:Olive Oil 4:1 (AOO) was not used due to lack of solubility of test article. In our lab, the DMSO vehicle has produced consistent results with the positive control 25% HCA, as well as with other sensitizers and non-sensitizers. The test article dose concentrations were proposed by MB and approved by the sponsor to approach but not exceed the irritation threshold. The dosing schedule follows:

Dose Groups:

Test Substance	Animal #'s
Naive	1-5
Vehicle – (DMSO)	6-10
Positive Control – (25% HCA)	11-15
25% MTDID 6675,	31-35
50% MTDID 6675,	36-40
100% MTDID 6675,	41-45

Type and Frequency of Observations:

All animals in the study were observed once daily throughout the study for clinical signs, either of local irritation at the application site or of systemic toxicity, and for mortality. Body weights were recorded on Day 1, immediately prior to dosing, and on Day 6, prior to BrdU injection. Ear measurements were taken from all animals and recorded on Days 1, 3, and 6.

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EXPERIMENTAL DESIGN (cont'd)

Node Isolation and Processing:

On Day 6, approximately 120 hours following the initial dose, and five hours prior to sacrifice, the mice were injected with 5-bromo-2'-deoxy-uridine (BrdU), in Dulbecco's Phosphate Buffered Saline (DPBS) at a dose of approximately 150 mg/kg. The BrdU was administered by intraperitoneal injection using a 27-gauge needle. This thymidine analog becomes incorporated into the DNA of proliferating cells, including proliferating nodal lymphocytes. At sacrifice, the auricular lymph nodes were isolated (see Appendix D for lymph node location) and single-cell suspensions were generated in Fetal Bovine Serum (FBS) and fixed with 85% ethanol. The cell suspensions were used to determine BrdU incorporation into the lymphocyte DNA (percentage of proliferating BrdU+ LNC).

Flow Cytometry:

Flow cytometric analyses were conducted using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an Omnichrome argon laser emitting at 488 nm with 15 mW of power. For DNA and/or BrdU determinations, clumps of nuclei were excluded from analysis using gates set on integrated red fluorescence signals. The histograms generated in these experiments were analyzed using Cell Quest Software (Becton Dickinson Immunocytometry Systems, San Jose CA).

Determination of Stimulation Index:

Proliferation of Lymphocytes (# of BrdU + cells): Measured aliquots of fixed cells were washed and resuspended in 1 N HCl containing 0.5% Triton X-100. This acid denaturation step allows the BrdU antibody to quantitatively interact with BrdU that has been incorporated into the cellular DNA. Following a one-hour denaturation period, the samples were neutralized by washing with sodium tetraborate (pH 8.5). The nuclei were then washed with a staining buffer (1.0% Bovine Serum Albumin, 0.03% sodium azide and 0.5% Tween-20 in Phosphate buffered saline (PBS), pH 7.4) and incubated with the BrdU-specific (fluorescein-conjugated) antibody (Becton Dickinson Immunocytometry Systems, San Jose CA 95131, USA). The nuclei were again washed with the staining buffer, resuspended in DPBS containing the DNA-specific dye propidium iodide and the percentage of nuclei staining positive for BrdU (i.e., proliferating cells in "S" phase) was determined using flow cytometry.

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EXPERIMENTAL DESIGN (cont'd)

Cell Number Determination: For cell number determination for each animal, a measured aliquot of fixed cells was transferred to a tube containing propidium iodide. The number of cells in each aliquot (representing a 1/500 dilution of the LNC suspension) was determined by flow cytometry.

The total number of cells in the lymph node was determined by sampling an aliquot that represented 1/500 (dilution factor = 500) of the total number of cells in the node (Equation 1). To determine the number of BrdU+ cells in the lymph node, the total number of LNCs counted in an aliquot of the cell suspension was multiplied by the percentage of LNCs that incorporated BrdU (Equation 2).

Equation 1: (Mean # of Cells in Aliquot) X (Dilution Factor) = **Total # of Cells in Node**

Equation 2: (Total # of Cells in Node) X $\frac{\text{(BrdU+ Cells)\%}}{100\%}$ = **# Proliferating Lymphocytes**

The means and standard deviations were calculated for each dose group.

Analysis of Stimulation Index Data:

Calculation of Stimulation Index (SI): For each animal, lymph node cell proliferation as measured by the number of Proliferating Lymphocytes was determined by flow cytometry and the mean ± SD was then calculated for each group. The mean number of Proliferating Lymphocytes in each animal was then divided by the mean number of Proliferating Lymphocytes in the vehicle control group. (For the 100% test article group, the mean number of Proliferating Lymphocytes was divided by the mean number of BrdU+ LNC in the naive control group since no DMSO was used in the dose preparation for this group.) This "Test/ Control Ratio" is the "Stimulation Index" (SI) and was calculated for each animal according to Equation 3 below:

Equation 3:

$$\frac{\text{Mean \# of Proliferating Lymphocytes per animal}}{\text{Mean \# of Proliferating Lymphocytes from vehicle control group}} = \text{Stimulation Index (SI) per animal}$$

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EXPERIMENTAL DESIGN (cont'd)

EC3 calculation:

Calculation of EC3: The concentration at which SI = 3 (EC3) was calculated according to the following[1]:

Equation 4: $EC3 = c+[(3-d)/(b-d)]x(a-c)$

where a, c are the concentrations of the Test Article and b, d are SI values at each respective concentration.

The skin sensitization potency can be classified according to the following table[2,3]:

Category	EC3(%)
Extreme	<0.1
Strong	≥0.1-<1
Moderate	≥1-<10
Weak	≥10-≤100

[1] D.A.Basketter, et al., "A Comparison of Statistical Approaches to the Derivation of EC3 values from Local Lymph Node Assay Dose Responses", J App Tox (1999), 19:261-266.

[2] G. F. Gerberick, C. A. Ryan, P. S. Kern, R. J. Dearman, I. Kimber, G. Y. Patlewicz, and D. A. Basketter. A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. *Contact Dermatitis* 50 (5):274-288, 2004.

[3] ECETOC. Contact Sensitisation: Classification According to Potency. Brussels:European Centre for Ecotoxicology and Toxicology of Chemicals. ECETOC Technical Report No. 87. 2003.

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EXPERIMENTAL DESIGN (cont'd)

Immunophenotyping

Aliquots of LNC (approximately 5×10^5 cells) were stained with both fluorescently-labeled anti-mouse B220 antibody (B cells) and anti-mouse CD3 antibody (T cells) in PBS-based buffer using an appropriate titer (BD Biosciences). Cells were then washed and resuspended in PBS containing the viability dye 7AAD. Live cells were analyzed by flow cytometry to determine % B cells, % T cells, and the B:T cell ratio. The mean values for each parameter were calculated for each test group and compared to the vehicle control group. Values for the 100% test article group were also compared to the naïve control group.

Analysis of Immunophenotype Data

Immunophenotype analysis was performed by calculating the mean %B220 and %CD3 LNC for each test group and then calculating the percentage change in %B220 versus the vehicle control group (Equation 5). The B:T cell ratio (Equation 6) was calculated by dividing the mean %B cells by the mean %T cells for each group.

Equation 5: %B220+ (B cells)

$$\frac{\text{Mean \%B220+ LNC from Treated Group}}{\text{Mean \%B220+ LNC from Vehicle Control Group}} = \text{fold change \%B220+ cells}$$

Equation 6: B:T cell ratio for each group is calculated by:

6A:

$$\frac{\text{Mean \%B220+ LNC from Treated Group}}{\text{Mean \%CD3+ LNC from Vehicle Control Group}} = \text{B:T cell ratio}$$

6B:

$$\frac{\text{B:T cell ratio from Treated Group}}{\text{B:T cell ratio from Vehicle Control Group}} = \text{fold change B:T Ratio}$$

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EXPERIMENTAL DESIGN (cont'd)

Interpretation of the Data:

A test article is considered to have a sensitizing potential if treatment results in a 3-fold or greater increase in the LNC proliferation (as shown by total BrdU+ cells) relative to that obtained for the vehicle control. Therefore, test articles that yield a SI ≥ 3 are characterized as potential sensitizing substances If a test article has an SI>3 and significant ear swelling or other data indicating irritation, immunophenotyping data can be used to determine whether that test article is a sensitizer or irritant. For immunophenotyping, increases in the %B cells or B:T ratio of ≥ 1.25-fold (>25% over vehicle control) are considered predictive of a true sensitizer.

Retention of the Data:

Upon signing the final report, all raw data, supporting documentation and reports are submitted to the Archivist by the Study Director. The raw data is filed at MB Research by project number. The final report is filed at MB Research by sponsor name and MB project number.

The test article will be returned to the submitter following submission of the report.

Amendment of the Protocol:

EC3 was calculated for the test article and included in the report. The EC3 value is a representative of potency. See Appendix L for protocol in its entirety.

Deviations to the Good Laboratory Practices

Although complete test article characterization was not provided to the study director prior to study initiation, information on the identity, storage, purity, composition and uniformity was provided. In addition, the test article characterization, provided by the sponsor prior to study initiation, was not conducted under the Good Laboratory Practices. Because of the short duration, the information provided is considered adequate for the intents and purposes of this study.

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RESULTS AND DISCUSSION

Test Article Formulation and Dosing:

The test article formed a homogenous mixture in the vehicle, DMSO. No difficulties were experienced with the application of the test articles to the ears or with the retention of test articles by the ear surface.

Body Weights (Appendix A):

Body weight changes were normal, except for the animal in the 100% dose group, which lost 1.4 grams and appeared emaciated.

Mortality and Systemic Observations (Appendix B):

All animals survived the in-life phase of the study and appeared normal, except for one animal in the 100% dose group, which appeared emaciated and exhibited a wet anogenital area on Day 6 of the definitive study. This animal could not be injected with BrdU, so lymph nodes were not harvested. The signs observed in this animal are not believed to be test article-related since all other animals in this group appeared normal.

Ear Swelling (Table 1, Appendices C & D):

A preliminary dermal irritation screen was performed and ear swelling measurements were used to assess irritation potential (See Appendix C for data and Appendix D for diagram). Ear thickness measurements were performed on Day 1 prior to dosing, on Day 3 before the third test article application, and on Day 6 before sacrifice. None of the concentrations tested resulted in increases in ear thickness greater than 25%, and therefore was not considered irritating. Based on these preliminary dermal irritation screen results and test article solubility data, concentrations of 25%, 50% and 100% of the test article were used for the definitive Local Lymph Node Assay. These concentrations corresponded to approximately 7.5%, 15% and 29.9% of the active ingredient in the test article.

In the definitive study, none of the MTDID 6675, concentrations tested resulted in dermal irritation. The positive control, 25% HCA, had a greater than 25% increase in ear swelling on Day 6, which is consistent with the literature historical results and based on our general experience occurs in about 90% of studies when using 25% HCA in the vehicle DMSO. This result is considered mildly to moderately irritating.

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RESULTS AND DISCUSSION (cont'd)

Table 1: Ear Swelling

Treatment	Mean Ear Thickness (mm)			Change (%)	
	Pre-dosing (Day 1)	48 Hr (Day 3)	End In-Life (Day 6)	Day 3 – Day 1	Day 6 – Day 1
Naive	0.18	0.18	0.18	0.0%	0.0%
DMSO (Vehicle)	0.19	0.20	0.20	5.3%	5.3%
25% HCA (Positive Control)[b]	0.19	0.24	0.26	26.3%	36.8%[a]
25% MTDID 6675,	0.19	0.20	0.20	5.3%	5.3%
50% MTDID 6675,	0.18	0.20	0.20	11.1%	11.1%
100% MTDID 6675,	0.19	0.19	0.20	0.0%	5.3%

a = a test substance leading to increases in ear thickness of greater than 25% is considered irritating
b = considered mildly to moderately irritating

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RESULTS AND DISCUSSION (cont'd)

Mean Number and Percent of Proliferating Lymph Node Cells (Table 2):

The total number of proliferating lymphocytes was calculated by multiplying total cell number by the percentage of the cells that have incorporated BrdU. The mean total lymphocyte proliferation for each of the treatment groups is noted in Table 2.

Table 2: Mean Number of Proliferating Cells in the Lymph Nodes of each Treatment Group

Treatment	Vehicle	Mean LNC # ($x10^3$)	% BrdU+ LNC	Lymphocyte Proliferation (#BrdU+)
Naive	—	1463	0.36	5100
DMSO (Vehicle)	—	2560	0.61	11929
25% HCA (Positive Control)	DMSO	12462	1.48	183010
25% MTDID 6675.	DMSO	798	1.27	9824
50% MTDID 6675.	DMSO	1193	2.39	28265
100% MTDID 6675.	DMSO	1281	3.80	45277

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RESULTS AND DISCUSSION (cont'd)

Determination of the Stimulation Index (SI) (Table 3, Appendices E, F & I):

Stimulation indices were calculated by dividing the total number of proliferating lymphocytes for each animal by the mean number of proliferating lymphocytes in the vehicle-exposed group. For the 100% test article group, the total lymphocyte proliferation was divided by that of the naive control group; for comparison, the SI for the 100% test article group was also calculated based on the vehicle control group. As the naïve group is the control for the 100% test article group, the calculated SI for this group was 1.0. The mean SI and standard deviation for each treatment group was then calculated (Table 3). An SI ≥ 3 indicates a potential sensitizing response. (See Appendix E for individual data and Appendix F for graph).

Table 3: Stimulation Index

Treatment	SI	S.D.
Naive	1.0	(+/- 0.6)
DMSO (Vehicle)	1.0	(+/- 0.5)
25% HCA (Positive Control)	15.3[a]	(+/- 2.4)
25% MTDID 6675,	0.8	(+/- 0.1)
50% MTDID 6675,	2.4	(+/- 1.4)
100% MTDID 6675, (fold to naive)	8.9[a]	(+/- 5.6)
100% MTDID 6675, (fold to vehicle)	3.8[a]	(+/- 2.4)

a = SI ≥ 3 indicates a potential sensitizing response

Statistical evaluation of the calculated SI values were made by Student's t-test, using GraphPad InStat™, c.1990-1994, v 2.05a+ (See Appendix I). However, determination of test article dermal sensitization was based on the criterion that test articles that yield a SI ≥ 3 are characterized as potential sensitizing substances.

EC3 calculation:

The EC3 was calculated to be 54.8%. This corresponds to a concentration of 16.4% of the active ingredient in the test article.

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RESULTS AND DISCUSSION (cont'd)

Immunophenotyping of Lymph Node Cells – B and T cells (Tables 4 & 5, Appendices G & H):

The %B cells, %T cells, and B:T cell ratio were determined by flow cytometry as described in the Experimental Design section of this report. Group means and standard deviations are tabulated below. (See Appendix G for individual data and Appendix H for graphs).

Table 4: %B220+ (B Cells), % CD3+ (T Cells)

Treatment	% B220 (B Cells) Mean	S.D.	Fold compared to vehicle	% CD3 (T Cells) Mean	S.D.
Naive	13.9	(+/- 4.6)	—	70.5	(+/- 5.7)
DMSO (Vehicle)	11.8	(+/- 2.7)	—	73.0	(+/- 6.0)
25% HCA (Positive Control)	27.1	(+/- 2.8)	2.30#	58.3	(+/- 1.5)
25% MTDID 6675,	12.3	(+/- 1.8)	1.05	73.2	(+/- 3.8)
50% MTDID 6675,	14.3	(+/- 1.6)	1.22	72.9	(+/- 2.8)
100% MTDID 6675, (fold to Naive)	22.4	(+/- 3.0)	1.62	62.6	(+/- 4.3)
100% MTDID 6675, (fold to Vehicle)	22.4	(+/- 3.0)	1.91#	62.6	(+/- 4.3)

= greater than 1.25-fold (≥25% increase) over vehicle control = positive sensitizing response

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RESULTS AND DISCUSSION (cont'd)

Table 5: B:T Lymphocyte Cell Ratio

Treatment		Mean S.D.	Fold* compared to Vehicle
Naive		0.20 (+/- 0.09)	—
DMSO (Vehicle)		0.16 (+/- 0.05)	—
25% HCA (Positive Control)		0.46 (+/- 0.05)	2.84#
25% MTDID 6675,		0.17 (+/- 0.03)	1.04
50% MTDID 6675,		0.20 (+/- 0.03)	1.20
100% MTDID 6675,	(fold to Naive)	0.36 (+/- 0.07)	1.79
100% MTDID 6675,	(fold to Vehicle)	0.36 (+/- 0.07)	2.21#

= greater than 1.25-fold (≥25% increase) over vehicle control = positive sensitizing response

The test article elicited a fold increase in %B cells of 1.05 at 25%, 1.22 at 50% and 1.91 at 100%, compared to the vehicle control, and an increase of 1.62-fold at 100% compared to the naive control. Fold increases in B:T ratio were 1.04 at 25%, 1.20 at 50%, and 2.21 at 100%, compared to vehicle, and 1.79 at 100% compared to the Naive control.

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CONCLUSION

Topical application of the test article MTDID 6675, , at 100% (29.9% as the active ingredient) resulted in a stimulation index greater than 3 (SI > 3), as well as a >25% increase in %B cells and in the B:T ratio compared to both vehicle and naive controls. Therefore, this test article is a dermal sensitizer in the Local Lymph Node Assay. The test article has a calculated EC3 value of 54.8% (16.4% as the active ingredient). Based on ECETOC criteria, the active ingredient in MTDID 6675, , can be classified as a weak sensitizer.

FINAL REPORT

Approved by: Melissa Kirk 10-25-07
Melissa Kirk, Ph D — Date
Study Director

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MB Research Laboratories

Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

Appendix A: Body Weights in grams

Naive

An. #	Sex	Day 1	Day 6	B.W. Change
1	F	19.8	20.9	1.1
2	F	17.5	18.3	0.8
3	F	19.7	21.1	1.4
4	F	21.1	22.6	1.5
5	F	21.5	22.5	1.0
	MEAN	19.9	21.1	1.2
	S.D	1.8	1.7	0.3
	#	5	5	5

DMSO - Vehicle

An. #	Sex	Day 1	Day 6	B.W. Change
6	F	22.0	22.4	0.4
7	F	20.0	21.9	1.9
8	F	20.0	21.3	1.3
9	F	21.2	23.2	2.0
10	F	19.5	21.5	2.0
	MEAN	20.6	22.1	1.5
	S.D.	1.0	0.8	0.7
	#	5	5	5

25% HCA - Positive Control

An. #	Sex	Day 1	Day 6	B.W. Change
11	F	19.2	20.1	0.9
12	F	21.3	22.9	1.6
13	F	19.3	20.8	1.5
14	F	20.9	22.7	1.8
15	F	20.6	20.4	-0.2
	MEAN	20.3	21.4	1.1
	S.D.	1.0	1.3	0.8
	#	5	5	5

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MB Research Laboratories

Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

Appendix A (cont'd): Body Weights in grams

25% MTDID 6675,

An. #	Sex	Day 1	Day 6	B.W. Change
31	F	22.2	24.3	2.1
32	F	21.4	22.7	1.3
33	F	20.7	22.2	1.5
34	F	21.4	22.1	0.7
35	F	19.7	19.6	-0.1
	MEAN	21.1	22.2	1.1
	S.D.	0.9	1.7	0.8
	#	5	5	5

50% MTDID 6675,

An. #	Sex	Day 1	Day 6	B.W. Change
36	F	19.3	21.8	2.5
37	F	18.9	21.1	2.2
38	F	16.7	19.0	2.3
39	F	17.6	19.6	2.0
40	F	21.5	24.4	2.9
	MEAN	18.8	21.2	2.4
	S.D.	1.8	2.1	0.3
	#	5	5	5

100% MTDID 6675,

An. #	Sex	Day 1	Day 6	B.W. Change
41	F	20.1	23.5	3.4
42	F	21.0	19.6	-1.4
43	F	20.0	21.7	1.7
44	F	19.9	20.9	1.0
45	F	20.8	22.1	1.3
	MEAN	20.4	21.6	1.2
	S.D.	0.5	1.4	1.7
	#	5	5	5

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Appendix B: Systemic Observations

Naive

TIME	ANIMAL #/SEX				
PERIODS	1/F	2/F	3/F	4/F	5/F
Day 1					
Day 2					
Day 3					
Day 4					
Day 5					
Day 6					

DMSO - Vehicle

TIME	ANIMAL #/SEX				
PERIODS	6/F	7/F	8/F	9/F	10/F
Day 1					
Day 2					
Day 3					
Day 4					
Day 5					
Day 6					

25% HCA - Positive Control

TIME	ANIMAL #/SEX				
PERIODS	11/F	12/F	13/F	14/F	15/F
Day 1					
Day 2					
Day 3					
Day 4					
Day 5					
Day 6					

No entry indicates animal appeared normal at that observation period.

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MB Research Laboratories

Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

Appendix B (cont'd): Systemic Observations

50% MTDID 6675, I

TIME	ANIMAL # / SEX				
PERIODS	31/F	32/F	33/F	34/F	35/F
Day 1					
Day 2					
Day 3					
Day 4					
Day 5					
Day 6					

100% MTDID 6675,

TIME	ANIMAL # / SEX				
PERIODS	36/F	37/F	38/F	39/F	40/F
Day 1					
Day 2					
Day 3					
Day 4					
Day 5					
Day 6					

MTDID 6675,

TIME	ANIMAL # / SEX				
PERIODS	41/F	42/F	43/F	44/F	45/F
Day 1					
Day 2					
Day 3					
Day 4					
Day 5					
Day 6		R, W, 1			

No entry indicates animal appeared normal at that observation period
R = Anogenital area wet
W = Appears emaciated
1 = Red substance at injection site

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Appendix C: Primary Dermal Irritation Dose Range Finder (Ear Swelling Screen)

Treatment	Mean Ear Thickness (mm)			Change (%)	
	Pre-dosing (Day 1)	48 Hr (Day 3)	End In-Life (Day 6)	Day 3 – Day 1	Day 6 – Day 1
25% MTDID 6675,	0.19	0.19	0.19	0.0%	0.0%
50% MTDID 6675,	0.20	0.21	0.21	5.0%	5.0%
100% MTDID 6675,	0.20	0.20	0.19	0.0%	-5.0%

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MB Research Laboratories

Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

Appendix D: Location of Draining Lymph Nodes

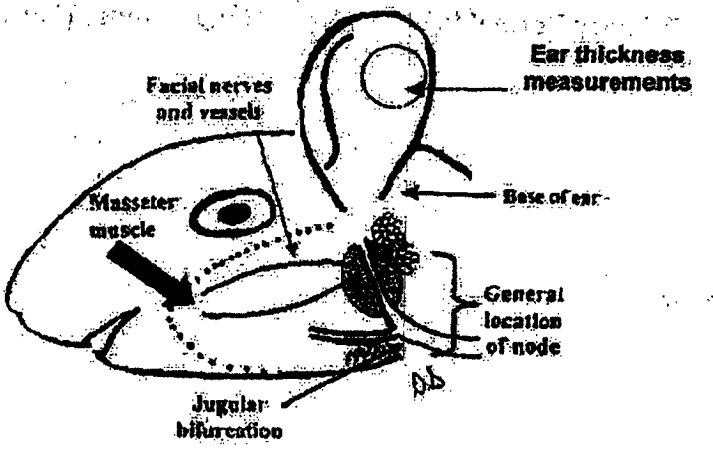

Figure 1. Lateral view

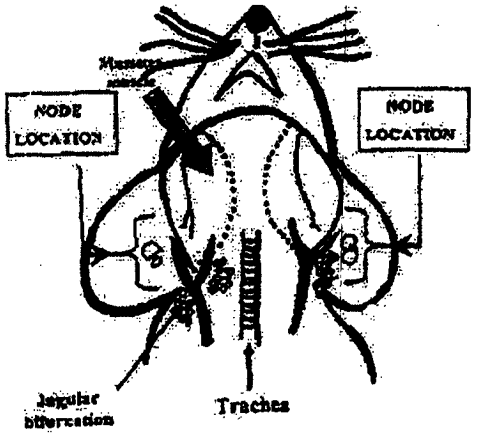

Figure 2. Ventral view

From D. Sailstad in *The Murine Local Lymph Node Assay: a Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds.*, NIEHS and ICCVAM 1994

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MB Research Laboratories

Study Title : LLNA
Project # : MB 07-15981.26
Protocol : 5650M

Appendix E: Stimulation Index

Treatment	Animal#	Total # Cells in Node x10^3	%BrdU+	Lymphocyte Proliferation	SI
Naïve	1	1486	0.21	3120	0.6
	2	463	0.48	2220	0.4
	3	2658	0.39	10366	2.0
	4	1435	0.31	4449	0.9
	5	1273	0.42	5346	1.0
	Mean	1463	0.36	5100	**1.0**
	StDev	785	0.10	3179	0.6
DMSO (Vehicle)	6	2608	0.31	8086	0.7
	7	2368	0.52	12315	1.0
	8	3829	0.56	21440	1.8
	9	641	0.79	5066	0.4
	10	3352	0.38	12739	1.1
	Mean	2560	0.51	11929	**1.0**
	StDev	1220	0.19	6187	0.5
25% HCA (Positive Control)	11	12012	1.38	166766	13.9
	12	15215	1.37	208446	17.5
	13	12142	1.80	218559	18.3
	14	11614	1.40	162598	13.6
	15	11325	1.41	159683	13.4
	Mean	12462	1.48	183010	**15.3a**
	StDev	1573	0.18	28146	2.4
25% MTDID 6675,	31	771	1.48	11407	1.0
	32	665	1.51	10034	0.8
	33	711	1.04	7394	0.6
	34	1076	0.86	9254	0.8
	35	766	1.44	11030	0.9
	Mean	798	1.27	9824	**0.8**
	StDev	162	0.30	1600	0.1
50% MTDID 6675,	36	2298	2.37	54451	4.6
	37	601	2.88	17309	1.5
	38	1228	2.26	27742	2.3
	39	469	2.11	9885	0.8
	40	1371	2.33	31938	2.7
	Mean	1193	2.39	28265	**2.4**
	StDev	729	0.29	17010	1.4

a = SI≥3

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Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

Appendix E: Stimulation Index (cont'd)

Treatment	Animal#	Total # Cells in Node x10^3	%BrdU+	Lymphocyte Proliferation	SI
100% MTDID 6675,	41	990	3.19	31589	6.2
	42	Z	Z	Z	Z
	43	1982	3.36	66578	13.1
	44	1911	3.72	71071	13.9
	45	242	4.91	11870	2.3
	Mean	1281	3.80	45277	8.9a
	StDev	827	0.78	28416	5.6

a = SI≥3

Z = Animal #42 was too sick to be injected with BrdU. It was sacrificed with the rest of the group, but nodes were not taken.

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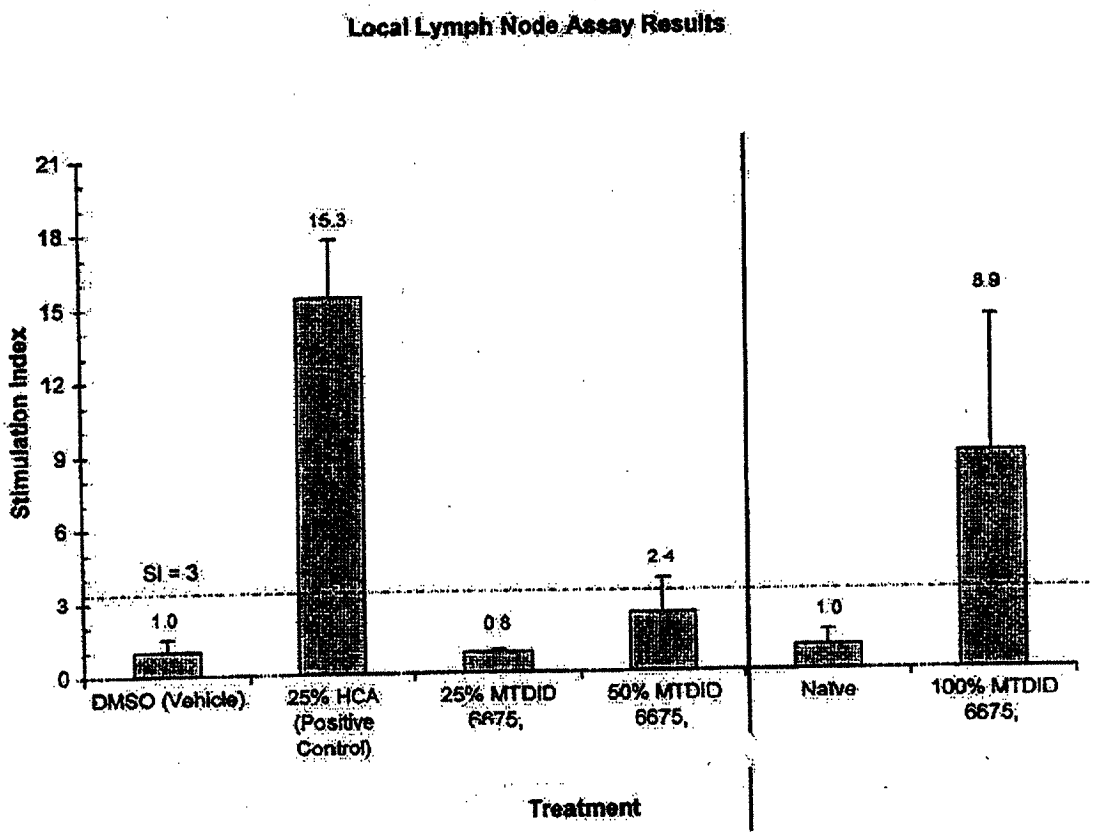
Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

Appendix F: Stimulation Index vs. Treatment



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Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

Appendix G: Immunophenotyping – B and T cells

Treatment	Animal	% B	% T	B:T Ratio
Naive	1	12.43	74.43	0.17
	2	21.62	61.54	0.35
	3	13.38	68.12	0.20
	4	9.26	74.56	0.12
	5	12.66	73.79	0.17
	Mean	**13.87**	**70.49**	**0.20**
	St Dev	4.61	5.67	0.09
DMSO (Vehicle)	6	10.21	78.95	0.13
	7	9.25	74.91	0.12
	8	11.44	68.12	0.17
	9	16.26	67.30	0.24
	10	11.71	75.58	0.15
	Mean	**11.77**	**72.97**	**0.16**
	St Dev	2.70	5.05	0.05
25% HCA (Positive Control)	11	29.72	58.78	0.51
	12	26.46	56.19	0.47
	13	23.40	60.41	0.39
	14	30.10	58.32	0.52
	15	25.59	57.65	0.44
	Mean	**27.05**	**58.27**	**0.46**
	St Dev	2.84	1.55	0.05
25% MTDID 6675,	31	12.48	70.16	0.18
	32	11.17	72.83	0.15
	33	14.84	68.87	0.22
	34	12.40	75.98	0.16
	35	10.84	77.92	0.14
	Mean	**12.35**	**73.15**	**0.17**
	St Dev	1.57	3.81	0.03
50% MTDID 6675,	36	12.47	76.42	0.16
	37	13.65	72.02	0.19
	38	15.32	68.83	0.22
	39	13.59	73.98	0.18
	40	16.64	73.30	0.23
	Mean	**14.31**	**72.91**	**0.20**
	St Dev	1.61	2.79	0.03

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MB Research Laboratories

Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

Appendix G: Immunophenotyping – B and T cells (cont'd)

100% MTDID 6675,	41	25.87	61.83	0.42
	42	Z	Z	Z
	43	20.54	66.85	0.31
	44	23.93	56.95	0.42
	45	19.44	64.63	0.30
	Mean	**22.46**	**62.57**	**0.36**
	St Dev	2.98	4.27	0.07

Z = Animal #42 was too sick to be injected with BrdU. It was sacrificed with the rest of the group, but nodes were not taken.

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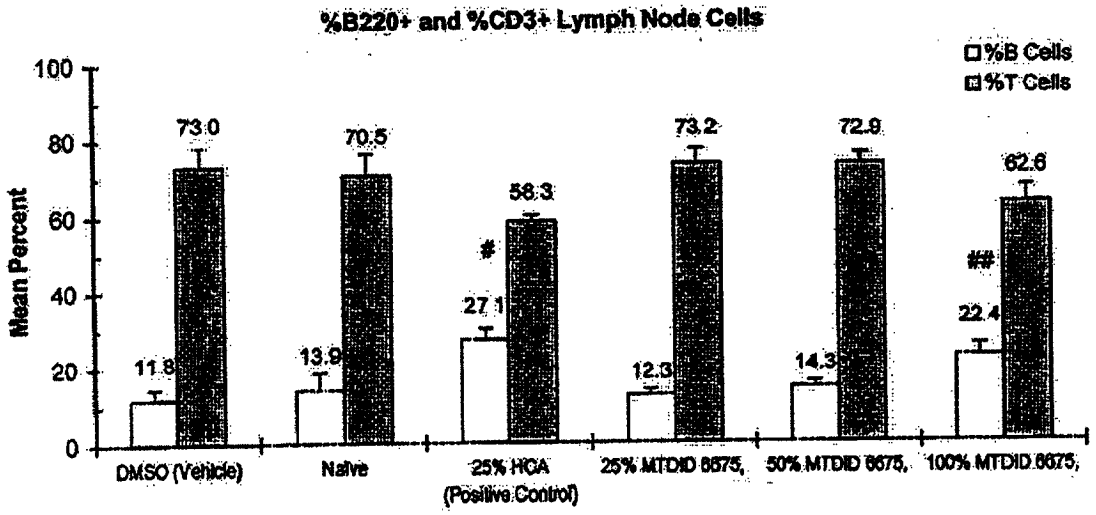
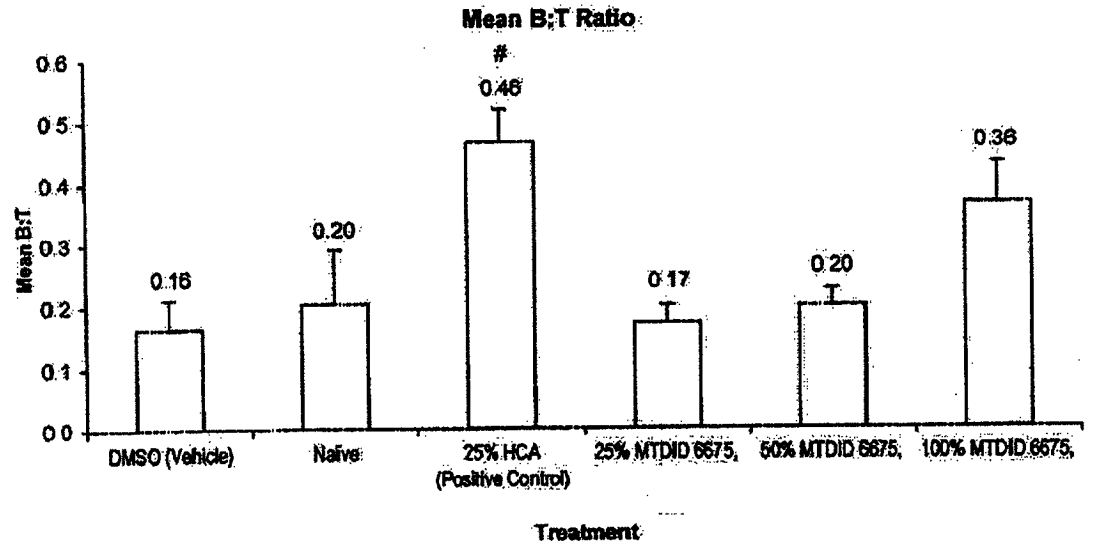
MB Research Laboratories

Study Title : LLNA
Project # : MB 07-15961.26
Protocol : 5650M

Appendix H: Immunophenotyping – B and T cells vs. Treatment





= greater than 1.25-fold (≥25% increase) over vehicle control = positive sensitizing response
= greater than 1.25-fold (≥25% increase) over naive control = positive sensitizing response

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Appendix I: Statistical Analysis

Comparison of SI values by Student's t-Test	Mean Difference	P Value
DMSO (Vehicle) vs. 25% HCA (Positive Control)	-14.340	***
DMSO (Vehicle) vs. 25% MTDID 6675,	-0.1800	ns
DMSO (Vehicle) vs. 50% MTDID 6675,	-1.380	ns

Comparison of SI values by Student's t-Test	Mean Difference	P Value
Naive vs. 100% MTDID 6675,	-7.895	***

*** = statistically significant (99.9% confidence)
ns = not statistically significant (p>0.05)

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MB Research Laboratories

Study Title : LLNA
Project # : MB 07-16961.26
Protocol : 5650M

QUALITY ASSURANCE EVALUATION

The Quality Assurance Unit has inspected a critical phase of this study, audited the raw data and the report and determined that the methods and results contained herein accurately reflect the raw data. No changes/modifications from the approved protocol or Standard Operating Procedures were made without proper authorization and documentation. A summary of the compliance inspections is presented below.

Date of Inspection	Phase	Performed By	Date Findings Reported to Mgmt.	Sty. Dir.
07/30/07	BrdU injection dose	Katrina Hanson	10/24/07	10/25/07
08/16/07	Raw data audit	Erin Range	10/24/07	10/25/07
09/11/07	Draft report audit	William J. Kintigh	10/24/07	10/25/07
10/04/07	Second draft report audit	William J. Kintigh	10/24/07	10/25/07
10/24/07	Final report audit	William J. Kintigh	10/24/07	10/25/07

William J. Kintigh, B.S.,
Quality Assurance Unit

25 Oct 2007
Date

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Appendix J
MB 07-15961 26

The Jackson Laboratory

Animal Health Report

AREA: AX-9 **Report # 0307AX9**

Check our web site for recent updates to this report: http://jaxmice.jax.org/health/ax9.pdf

OVERALL HEALTH STATUS

☐ Excellent (no positive results) ■ Superior (no pathogens, parasites or opportunists) ☐ Good (no pathogens or parasites)

AX-9 IS OPERATED AS A:

☐ Maximum barrier ☐ High Barrier ■ Standard barrier ☐ Research Standard barrier ☐ Low barrier

AX-9 IS IN:

■ Production Facility ☐ Research Facility ☐ Other Area*

*Other areas house small production-type colonies maintained as in-house resources or sources of lower-demand mice for distribution.

Mouse pathogens – NO POSITIVE RESULTS
These organisms are pathogenic for mice and/or have a significant potential for interference with research.
If any of these organisms are found in any JAX mouse, all shipments from the room are suspended and customers are notified.

Organism	Sample Tested	Test Method	Mar '07	Dec '06	Sep '06	Jun '06
VIRUSES						
Ectromelia virus	Serum	ELISA	0/20	0/21	0/21	0/20
GDVII (Theiler's) virus	Serum	ELISA	0/20	0/21	0/21	0/21
Hantaan virus	Serum	ELISA	0/20	0/21	0/21	0/21
K virus	Serum	ELISA	-	-	0/21	-
Lactic dehydrogenase elevating virus	Serum	Enzyme	0/10	-	-	-
Lymphocytic choriomeningitis (LCMV)	Serum	ELISA	-	0/21	-	0/21
Mouse minute virus (MMV)	Serum	ELISA	0/20	0/21	0/21	0/21
Mouse adenovirus (MAV)	Serum	ELISA	0/20	-	0/21	-
Mouse cytomegalovirus (MCMV)	Serum	ELISA	0/20	-	0/21	-
Mouse hepatitis virus (MHV)	Serum	ELISA	0/20	0/21	0/21	0/20
Mouse parvovirus (MPV)	Serum	ELISA	0/20	0/21	0/21	0/21
Mouse thymic virus (MTV)	Serum	IFA	0/20	-	0/20	-
Pneumonia virus of mice (PVM)	Serum	ELISA	0/20	0/21	0/21	0/20
Polyoma virus	Serum	ELISA	-	-	0/21	-
Reovirus 3 (REO 3)	Serum	ELISA	-	0/21	-	0/21
Rotavirus (EDIM)	Serum	ELISA	0/20	0/21	0/21	0/20
Sendai virus	Serum	ELISA	0/20	0/21	0/21	0/21
BACTERIA & MYCOPLASMA						
CAR bacillus	Serum	ELISA	-	0/21	-	0/21
Streptobacillus moniliformis	Trachea	Culture	0/05	0/06	0/06	0/06
Mycoplasma pulmonis	Serum	ELISA	0/20	0/21	0/21	0/21
Citrobacter rodentium	Intestine or feces	Culture	0/05	0/06	0/06	0/06
Salmonella spp.	Intestine or feces	Culture	0/05	0/06	0/06	0/06
Clostridium piliforme	Serum	ELISA	0/20	-	0/21	-

For additional details regarding our health

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Appendix J
MB 07-15961 26

Parasites – NO POSITIVE RESULTS

Although most of these organisms have limited potential to cause disease or interfere with research, they are unacceptable in most facilities. If any of these organisms are found, all shipments from the room are suspended and customers are notified.

Organism	Sample Tested	Test Method	Mar '07	Dec '06	Sep '06	Jun '06
Fleas, fur mites, lice	Fur	Visual	0/05	0/06	0/06	0/06
Follicle mites	Subcutis	Visual	0/05	0/06	0/06	0/06
Pinworms	Cecum	Visual	0/05	0/06	0/06	0/06
Roundworms and other helminths	Intestine	Visual	0/05	0/06	0/06	0/06
Tapeworms	Intestine	Visual	0/05	0/06	0/06	0/06
Encephalitozoon cuniculi	Serum	ELISA	-	0/21	-	0/21

Opportunistic organisms – NO POSITIVE RESULTS

These organisms are generally recognized as opportunistic pathogens that may complicate disease or interfere with research under certain circumstances, e.g., immunodeficient mice. They are not tolerated in any Production area – positive results trigger a clinical investigation designed to eliminate all infected mice. Shipping from the area is not stopped.

Organism	Sample Tested	Test Method	Mar '07	Dec '06	Sep '06	Jun '06
Bordetella bronchiseptica	Trachea	Culture	0/05	0/06	0/06	0/06
Corynebacterium kutscheri	Trachea	Culture	0/05	0/06	0/06	0/06
Helicobacter spp.	Intestine or feces	PCR	0/05	0/06	0/06	0/06
Klebsiella pneumoniae	Trachea, intestine, or feces	Culture	0/05	0/06	0/06	0/06
Murine norovirus (MNV)	Serum	ELISA	0/20	0/21	-	-
Pasteurella pneumotropica	Trachea	Culture	0/05	0/06	0/06	0/06
Pneumocystis carinii	Lung	PCR	-	-	-	-
Pseudomonas spp.	Intestine or feces	Culture	0/05	0/06	0/06	0/06
Staphylococcus aureus	Trachea	Culture	0/05	0/06	0/06	0/06
Streptococcus spp.	Trachea	Culture	0/05	0/06	0/06	0/06
Opportunistic protozoa (e.g., *Giardia*, *Spironucleus*)	Intestine	Micro	0/05	0/06	0/06	0/06

Other (nonpathogenic) organisms – Positive results for 1 organism

These organisms are generally viewed as normal flora of mice or as exogenous microorganisms with little or no potential to cause disease or interfere with research. If any of these organisms are found in any JAX mouse, the finding is noted in this report, but shipments are not suspended.

Organism	Sample Tested	Test Method	Mar '07	Dec '06	Sep '06	Jun '06
Klebsiella spp. other than *K. pneumoniae*	Trachea, intestine, or feces	Culture	3/05	3/06	4/06	2/06
Nonpathogenic protozoa (e.g., trichomonads)	Intestine	Micro	0/05	0/06	0/06	0/06

Peggy J. Danneman, VMD, MS, DACLAM
[illegible] Animal Health Services

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Appendix J
MB 07-15961 26

The Jackson Laboratory

Animal Health Report

AREA: MP-15 **Report #0507MP15**

Check our web site for recent updates to this report: http://jaxmice.jax.org/health/mp15.pdf

OVERALL HEALTH STATUS

☐ Excellent (no positive results) ■ Superior (no pathogens, parasites or opportunists) ☐ Good (no pathogens or parasites)

MP-15 IS OPERATED AS A:

☐ Maximum barrier ☐ High Barrier ■ Standard barrier ☐ Research Standard barrier ☐ Low barrier

MP-15 IS IN:

■ Production Facility ☐ Research Facility ☐ Other Area*

*Other areas house small production-type colonies maintained as in-house resources or sources of lower-demand mice for distribution.

Mouse pathogens – NO POSITIVE RESULTS

These organisms are pathogenic for mice and/or have a significant potential for interference with research.
If any of these organisms are found in any JAX mouse, all shipments from the room are suspended and customers are notified.

Organism	Sample Tested	Test Method	May '07	Feb '07	Nov '06	Aug '06
VIRUSES						
Ectromelia virus	Serum	ELISA	0/21	0/21	0/21	0/21
GDVII (Theiler's) virus	Serum	ELISA	0/21	0/21	0/21	0/21
Hantaan virus	Serum	ELISA	0/21	0/21	0/21	0/21
K virus	Serum	ELISA	-	-	0/21	-
Lactic dehydrogenase elevating virus	Serum	Enzyme	0/10	-	-	-
Lymphocytic choriomeningitis (LCMV)	Serum	ELISA	-	0/21	-	0/21
Mouse minute virus (MMV)	Serum	ELISA	0/21	0/21	0/21	0/21
Mouse adenovirus (MAV)	Serum	ELISA	0/21	-	0/21	-
Mouse cytomegalovirus (MCMV)	Serum	ELISA	0/21	-	0/21	-
Mouse hepatitis virus (MHV)	Serum	ELISA	0/21	0/21	0/21	0/21
Mouse parvovirus (MPV)	Serum	ELISA	0/21	0/21	0/21	0/21
Mouse thymic virus (MTV)	Serum	IFA	0/18	-	0/21	-
Pneumonia virus of mice (PVM)	Serum	ELISA	0/21	0/21	0/21	0/20
Polyoma virus	Serum	ELISA	-	-	0/21	-
Reovirus 3 (REO 3)	Serum	ELISA	-	0/21	-	0/21
Rotavirus (EDIM)	Serum	ELISA	0/21	0/21	0/21	0/21
Sendai virus	Serum	ELISA	0/21	0/21	0/21	0/21
BACTERIA & MYCOPLASMA						
CAR bacillus	Serum	ELISA	-	0/21	-	0/21
Streptobacillus moniliformis	Trachea	Culture	0/06	0/06	0/06	0/06
Mycoplasma pulmonis	Serum	ELISA	0/21	0/21	0/21	0/21
Citrobacter rodentium	Intestine or feces	Culture	0/06	0/06	0/06	0/06
Salmonella spp.	Intestine or feces	Culture	0/06	0/06	0/06	0/06
Clostridium piliforme	Serum	ELISA	0/21	-	0/21	-

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Appendix J
MB 07-15961.26

Parasites – NO POSITIVE RESULTS
Although most of these organisms have limited potential to cause disease or interfere with research, they are unacceptable in most facilities. If any of these organisms are found, all shipments from the room are suspended and customers are notified.

Organism	Sample Tested	Test Method	May '07	Feb '07	Nov '06	Aug '06
Fleas, fur mites, lice	Fur	Visual	0/06	0/06	0/06	0/06
Follicle mites	Subcutis	Visual	0/06	0/06	0/06	0/06
Pinworms	Cecum	Visual	0/06	0/06	0/06	0/06
Roundworms and other helminths	Intestine	Visual	0/06	0/06	0/06	0/06
Tapeworms	Intestine	Visual	0/06	0/06	0/06	0/06
Encephalitozoon cuniculi	Serum	ELISA	-	0/21	-	0/21

Opportunistic organisms – NO POSITIVE RESULTS
These organisms are generally recognized as opportunistic pathogens that may complicate disease or interfere with research under certain circumstances, e.g., immunodeficient mice. They are not tolerated in any Production area – positive results trigger a clinical investigation designed to eliminate all infected mice. Shipping from the area is not stopped.

Organism	Sample Tested	Test Method	May '07	Feb '07	Nov '06	Aug '06
Bordetella bronchiseptica	Trachea	Culture	0/06	0/06	0/06	0/06
Corynebacterium kutscheri	Trachea	Culture	0/06	0/06	0/06	0/06
Helicobacter spp.	Intestine or feces	PCR	0/06	0/06	0/06	0/06
Klebsiella pneumoniae	Trachea, intestine, or feces	Culture	0/06	0/06	0/06	0/06
Murine norovirus (MNV)	Serum	ELISA	0/21	0/21	0/21	-
Pasteurella pneumotropica	Trachea	Culture	0/06	0/06	0/06	0/06
Pneumocystis carinii	Lung	PCR	-	-	-	-
Pseudomonas spp.	Intestine or feces	Culture	0/06	0/06	0/06	0/06
Staphylococcus aureus	Trachea	Culture	0/06	0/06	0/06	0/06
Streptococcus spp.	Trachea	Culture	0/06	0/06	0/06	0/06
Opportunistic protozoa (e.g., *Giardia, Spironucleus*)	Intestine	Micro	0/06	0/06	0/06	0/06

Other (nonpathogenic) organisms – Positive results for 1 organism
These organisms are generally viewed as normal flora of mice or as exogenous microorganisms with little or no potential to cause disease or interfere with research. If any of these organisms are found in any JAX mouse, the finding is noted in this report, but shipments are not suspended.

Organism	Sample Tested	Test Method	May '07	Feb '07	Nov '06	Aug '06
Klebsiella spp. other than *K. pneumoniae*	Trachea, intestine, or feces	Culture	2/06	2/06	2/06	1/06
Nonpathogenic protozoa (e.g., trichomonads)	Intestine	Micro	0/06	0/06	0/06	0/06

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Appendix J
MB 07-15961 26

The Jackson Laboratory

Animal Health Report

AREA: AX-9 **Report # 0607AX9**

Check our web site for recent updates to this report: http://jaxmice.jax.org/health/ax9.pdf

OVERALL HEALTH STATUS

☐ Excellent (no positive results) ■ Superior (no pathogens, parasites or opportunists) ☐ Good (no pathogens or parasites)

AX-9 IS OPERATED AS A:

☐ Maximum barrier ☐ High Barrier ■ Standard barrier ☐ Research Standard barrier ☐ Low barrier

AX-9 IS IN:

■ Production Facility ☐ Research Facility ☐ Other Area*

*Other areas house small production-type colonies maintained as in-house resources or sources of lower-demand mice for distribution.

Mouse pathogens – NO POSITIVE RESULTS
These organisms are pathogenic for mice and/or have a significant potential for interference with research
If any of these organisms are found in any JAX mouse, all shipments from the room are suspended and customers are notified.

Organism	Sample Tested	Test Method	Jun '07	Mar '07	Dec '06	Sep '06
VIRUSES						
Ectromelia virus	Serum	ELISA	0/22	0/20	0/21	0/21
GDVII (Theiler's) virus	Serum	ELISA	0/22	0/20	0/21	0/21
Hantaan virus	Serum	ELISA	0/22	0/20	0/21	0/21
K virus	Serum	ELISA	-	-	-	0/21
Lactic dehydrogenase elevating virus	Serum	Enzyme	-	0/10	-	-
Lymphocytic choriomeningitis (LCMV)	Serum	ELISA	0/22	-	0/21	-
Mouse minute virus (MMV)	Serum	ELISA	0/22	0/20	0/21	0/21
Mouse adenovirus (MAV)	Serum	ELISA	-	0/20	-	0/21
Mouse cytomegalovirus (MCMV)	Serum	ELISA	-	0/20	-	0/21
Mouse hepatitis virus (MHV)	Serum	ELISA	0/22	0/20	0/21	0/21
Mouse parvovirus (MPV)	Serum	ELISA	0/22	0/20	0/21	0/21
Mouse thymic virus (MTV)	Serum	IFA	-	0/20	-	0/20
Pneumonia virus of mice (PVM)	Serum	ELISA	0/22	0/20	0/21	0/21
Polyoma virus	Serum	ELISA	-	-	-	0/21
Reovirus 3 (REO 3)	Serum	ELISA	0/22	-	0/21	-
Rotavirus (EDIM)	Serum	ELISA	0/22	0/20	0/21	0/21
Sendai virus	Serum	ELISA	0/22	0/20	0/21	0/21
BACTERIA & MYCOPLASMA						
CAR bacillus	Serum	ELISA	0/22	-	0/21	-
Streptobacillus moniliformis	Trachea	Culture	0/06	0/05	0/06	0/06
Mycoplasma pulmonis	Serum	ELISA	0/22	0/20	0/21	0/21
Citrobacter rodentium	Intestine or feces	Culture	0/06	0/05	0/06	0/06
Salmonella spp.	Intestine or feces	Culture	0/06	0/05	0/06	0/06
Clostridium piliforme	Serum	ELISA	-	0/20	-	0/21

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Parasites – NO POSITIVE RESULTS
Although most of these organisms have limited potential to cause disease or interfere with research, they are unacceptable in most facilities. If any of these organisms are found, all shipments from the room are suspended and customers are notified.

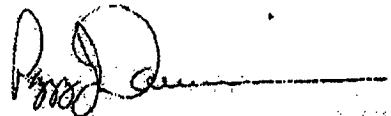
Organism	Sample Tested	Test Method	Jun '07	Mar '07	Dec '06	Sep '06
Fleas, fur mites, lice	Fur	Visual	0/06	0/05	0/06	0/06
Follicle mites	Subcutis	Visual	0/06	0/05	0/06	0/06
Pinworms	Cecum	Visual	0/06	0/05	0/06	0/06
Roundworms and other helminths	Intestine	Visual	0/06	0/05	0/06	0/06
Tapeworms	Intestine	Visual	0/06	0/05	0/06	0/06
Encephalitozoon cuniculi	Serum	ELISA	0/22	-	0/21	-

Opportunistic organisms – NO POSITIVE RESULTS
These organisms are generally recognized as opportunistic pathogens that may complicate disease or interfere with research under certain circumstances, e.g., immunodeficient mice. They are not tolerated in any Production area – positive results trigger a clinical investigation designed to eliminate all infected mice. Shipping from the area is not stopped.

Organism	Sample Tested	Test Method	Jun '07	Mar '07	Dec '06	Sep '06
Bordetella bronchiseptica	Trachea	Culture	0/06	0/05	0/06	0/06
Corynebacterium kutscheri	Trachea	Culture	0/06	0/05	0/06	0/06
Helicobacter spp	Intestine or feces	PCR	0/06	0/05	0/06	0/06
Klebsiella pneumoniae	Trachea, intestine, or feces	Culture	0/06	0/05	0/06	0/06
Murine norovirus (MNV)	Serum	ELISA	0/22	0/20	0/21	-
Pasteurella pneumotropica	Trachea	Culture	0/06	0/05	0/06	0/06
Pneumocystis carinii	Lung	PCR	-	-	-	-
Pseudomonas spp.	Intestine or feces	Culture	0/06	0/05	0/06	0/06
Staphylococcus aureus	Trachea	Culture	0/06	0/05	0/06	0/06
Streptococcus spp.	Trachea	Culture	0/06	0/05	0/06	0/06
Opportunistic protozoa (e.g., *Giardia*, *Spironucleus*)	Intestine	Micro	0/06	0/05	0/06	0/06

Other (nonpathogenic) organisms -- Positive results for 1 organism
These organisms are generally viewed as normal flora of mice or as exogenous microorganisms with little or no potential to cause disease or interfere with research. If any of these organisms are found in any JAX mouse, the finding is noted in this report, but shipments are not suspended.

Organism	Sample Tested	Test Method	Jun '07	Mar '07	Dec '06	Sep '06
Klebsiella spp. other than *K. pneumoniae*	Trachea, intestine, or feces	Culture	3/06	3/05	3/06	4/06
Nonpathogenic protozoa (e.g., trichomonads)	Intestine	Micro	0/06	0/05	0/06	0/06



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Appendix K
MB 07-15961 26

MB Research Laboratories

1765 Wentz Road
P.O. Box 178
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TEST ARTICLE CHARACTERIZATION INFORMATION

In compliance with Good Laboratory Practice (GLP) regulations, a characterization of the test article is required and should include identity, strength, purity, composition, stability and uniformity. This data must be reviewed by the Study Director prior to study initiation and will be included in the final report. (EPA 40 CFR 160.105 and 792.105; FDA 21 CFR 58.105; OECD 6.2).

In addition, the test article characterization should be performed in compliance with the Good Laboratory Practices.

Any exceptions to the GLP requirements will be indicated in the Compliance Statement of the final report.

Accordingly, please supply the following information for each test article submitted:

Test Article Identity	:	See attached CofA
Strength	:	"
Purity	:	"
Composition	:	"
Stability	:	No data available on this lot
Uniformity	:	Test article is a true solution

☐ This characterization was conducted under GLPs *(or)*

☒ This characterization was not conducted under GLPs

BY Steven C. Gordon (signature) FOR: 3M / Dyneon (company)

6/15/2007 (date)

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Appendix K
MB 07-15961.26

[redacted] Confidential

3M Materials Resource Division Analytical Laboratory

An ISO9001/2000 Certified Laboratory

Certificate of Analysis

Nominal Product:

Product Code: , MTDID #6675

Product Name:

Physical State: Clear and colorless aqueous solution

March 22, 2007

The sample of , MTDID #6675, was subjected to LC/MS and $^{19}F/^{1}H$-NMR internal standardization analyses to determine the absolute weight percent concentration of the nominal product and to characterize as many impurity components as possible. The qualitative and quantitative compositional results that were derived from the combined LC/MS and NMR analyses are summarized below.

TABLE-I

Sample: , MTDID #6675, aqueous

Compositional Results by LC/MS and ^{19}F-NMR Analyses

Identified Components [1,2]	^{19}F-NMR Absolute Wt.% Concentrations (duplicate trial measurements)
	29.9 ± 0.0%
	0.745 ± 0.007%
	0.0354 ± 0.0006%
	0.0012 ± 0.0002%

1 was assumed to be the cation for calculation purposes.

2 Trace levels of other unassigned components were also detected in the ^{19}F-NMR spectra.

Analytical Chemists:

3M MRD Analytical Laboratory Request

Page 1 of 1
File Reference:

TSCA SANITIZED

Appendix L
MB 07-15961.26

MB RESEARCH LABORATORIES
STANDARD PROTOCOL
5650M

1.0 TITLE OF STUDY: LOCAL LYMPH NODE ASSAY IN MICE (LLNA)

2.0 OBJECTIVE: To determine the sensitizing potential of topically applied test substances. This LLNA protocol, modified using flow cytometry analysis, is designed to be an alternative assay for the Buehler Guinea Pig Sensitization Assay defined in the ICCVAM report (63 CFR 37405-6, July 10, 1998) and the LLNA as defined in EPA OPPTS 870 2600, Final Guideline (March 2003), and OECD Test Guideline 429, effective April 2002.

3.0 TEST ARTICLE:

3.1: Source: All test articles will be supplied by the sponsor. Prior to initiation of the study, the sponsor should provide test article characterization to the Study Director that should include, if technically feasible, the name and quantities of unknown contaminants and impurities. Refer to section 13.4.3 of this protocol for additional information.

3.2: Label: Each test article will be identified by source, name, and/or code number, date of receipt at MB Research, and MB Project Number.

3.3: Storage: The test article will be stored at room temperature and humidity unless otherwise specified by the Sponsor.

3.4: Hazards: Based on the information provided by the Sponsor, appropriate routine safety precautions will be exercised in the handling of the test article.

3.5: Vehicle: As necessary, a suitable vehicle will be added to the test article to generate dilutions of the test article. The vehicle will be AOO (acetone:olive oil, 4:1) unless otherwise directed by the Sponsor. When a vehicle or diluent other than AOO will be used, it must be one that does not elicit any significant toxic effects and does not substantially alter the chemical or toxicological properties of the test article. Solubility testing and the use of vehicles other than AOO will be documented in the raw data.

4.0 GENERAL TEST SYSTEM PARAMETERS:

		IRRITATION PRESCREEN	QUANTITATIVE IRRITATION TEST (if needed)	MAIN TEST
4.1: Animal Requirements:				
4.1.1: Total Number of Animals	:	6	at least 12	at least 25
4.1.2: Number of Groups	:	at least 5, including one (1) control group receiving vehicle alone and (1) positive control group, plus at least three (3) test groups receiving consecutive concentrations		
4.1.3: No. Animals/Group	:	at least 5 (all female)		
4.1.4: Species/Strain	:	CBA/J or CBA/JHsd		
4.1.5: Age	:	8-12 weeks old at study initiation (age matched +/- one week)		

TSCA SANITIZED

Appendix L
MB 07-15961.26

4.2: Justification of Species/Strain and Number of Animals:

4.2.1: Species/Sex: LLNA uses female mice, the preferred experimental gender and species where there is the most detailed information available about the induction and regulation of immunological responses. The test guidelines specify females until gender-specific differences in the LLNA response are shown not to exist.

4.2.2: Strain: The protocol utilizes young adult (8-12 week old) female CBA strain mice. Female CBA/J, CBA/JHsd, or CBA/Ca strain mice are acceptable (as per OECD and EPA test guidelines) for use in the assay since, in several inter-laboratory validation studies, they displayed comparable responses. The source and strain used will be indicated in the study report.

4.2.3: Number of Animals:

4.2.3.1. Irritation Prescreen: The 6 mouse prescreen is the minimum number needed to determine if the test article has dermal irritation properties at the highest attainable concentration (maximum solubility) in the vehicle.

4.2.3.2. Quantitative Irritation Test: The optional Quantitative Irritation Test (12 mice) is used when irritation is present and the maximum acceptable dosing concentrations need to be determined.

4.2.3.3. Main Test: The minimum number of animals in the definitive test is 25, in 5 groups of 5 mice each. Occasionally, especially when dermal irritation is present, an additional 1-2 treatment groups (5-10 mice) may be necessary. For specialty vehicles or formulations, a naive group or a second vehicle group may be needed. The LLNA permits the reduction of animals required to assess the contact sensitizing activity of test substances compared to studies involving the use of guinea pigs. The minimum number per group recommended by ICCVAM and the EPA-OPPTS 870.2600 and OECD #429 test guidelines is five mice.

4.3: Husbandry:

4.3.1: Equilibration: The test animals will be conditioned to the housing facilities for at least five (5) days prior to study initiation.

4.3.2: Housing: Animals will be housed individually in suspended cages which conform to the size recommendations in the Guide for the Care and Use of Laboratory Animals DHEW (NIH). Absorbent white paper bedding, placed beneath the cage, will be changed at least three times per week. The animal room, reserved exclusively for mice, is temperature controlled and is equipped with a 12-hour light/dark cycle. Temperature and humidity will be continuously recorded using automatic recording devices.

4.3.3: Food: Fresh PMI (Diet #5001) will be available at all times.

TSCA SANITIZED

Appendix L
MB 07-15981.26

4.3.4: Water will be available at all times.

4.3.4.1: Analysis of Water and Acceptable Levels of Contaminants: Analysis of water is performed approximately four (4) times per year and results are compared against a list of acceptable levels of contaminants as provided by the water testing laboratory.

4.4: Control of Bias: From the available pool of animals, healthy female (must be nulliparous and non-pregnant) mice of the same age specified herein will be assigned to groups using standard accepted methods of randomization. The method for attaining the random numbers will be recorded in the study file.

4.4.1: Pre-study Body Weights: At the initiation of the study, the weight variation of test animals will not exceed ± 20 percent of the mean body weight.

4.5: Identification:

4.5.1: Cage: Each cage will be identified by a cage tag indicating the date of dosing, test article identification, MB project number, dose level, number and sex of animals.

4.5.2: Animal: Each animal will be identified by an indelible tail mark corresponding to the numbers documented on the data collection forms.

5.0 EXPERIMENTAL DESIGN:

5.1: Introduction: The LLNA determines the sensitization potential of a test substance by measuring the proliferation of lymphocytes in the auricular lymph nodes draining the site of exposure (ears). Lymphocyte proliferation will be measured by determining the incorporation of bromodeoxyuridine (BrdU) using a flow cytometer, a method shown to be equivalent to ^{3}T-thymidine-based measurements of lymphocyte proliferation.

5.2: Summary of Experimental Design:

LLNA PROTOCOL	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7+
7+ DAYS	T BW, ET	T	T ET	---	---	BrdU, BW, ET	P
						Sacrifice	

T = Topical application of test substance, vehicle or control

BrdU = At 5 hrs pre-sacrifice (t = -5 hrs), systemic administration of BrdU in PBS (200 µl per mouse; i.p.); at sacrifice, excision and processing of each mouse lymph node set (on an individual animal basis); preparation of a single-cell suspension of Lymph Node Cells (LNC)

BW = Body Weight

ET = Ear Thickness measured (digital micrometer or Peacock Dial thickness gauge) within 24 hrs pre-test, on Day 3 prior to dosing and pre-sacrifice on Day 6

P = Post in-life phase, ex-vivo flow cytometry procedures performed, measurement of BrdU incorporation into lymph node cells (LNC) analyzed, and Stimulation Index (SI) and other parameters calculated.

TSCA SANITIZED

Appendix L
MB 07-15961.26

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

5.3: Solvent/Vehicle Selection and Preparation: When preparing solutions, a suitable solvent vehicle will be selected from the following list (in order of preference) or according to instructions from the Sponsor. The default vehicle is AOO 4:1 (see Section 3.5). If AOO 4:1 is not useful as the vehicle, the secondary vehicles in section 5.3.1 will be investigated for solubility of the test article(s). Alternatively, a suitable vehicle may be chosen by the Sponsor in Section 13.1.2, based on the Sponsor's historical irritation and solubility data.

5.3.1: Optional LLNA Vehicles:

4:1 v/v Acetone/Olive oil (AOO)
Dimethyl sulfoxide (DMSO)
4:3:3 DMSO:Acetone:Ethanol (D_3AE 433)
Acetone
N,N-Dimethylformamide (DMF)
Dimethylacetamide (DMA)
4:3:3 Dimethylacetamide:Acetone:Ethanol (D_3AE 433)
Ethanol (50%, 95%, or 100%)
Methyl ethyl ketone (MEK), aka 2-Butanone
Ultra Pure Petrolatum
Propylene glycol (PG)

The preferred vehicle AOO is prepared by adding 4 parts (ml) of acetone for every 1 part (ml) olive oil. Wholly aqueous vehicles are to be avoided as per test guidelines. The vehicle will be labeled with identification, concentration, date of preparation, expiration date/(if applicable), storage/handling and the name/initials of the technician.

5.3.2: Vehicle Preference: Where possible the following vehicles should be used for the LLNA (in order of preference): AOO > Dimethylsulfoxide > D_3AE-433 (= D_3AE) > Acetone. If AOO is not to be used (or if a "clinically relevant solvent" or the "commercial formulation" into which the test article is added is to be used), the Sponsor will indicate this vehicle in section 13.1.2 of the SPONSOR REQUEST section of this protocol.

5.4: Positive Control: The moderate sensitizer alpha-hexyl cinnamic aldehyde (HCA, supplied by MB) at 25% or 50% in AOO (or suitable vehicle) will be used as the positive control as indicated by Sponsor in section 13. Additional positive controls such as the strong sensitizer 2,4-dinitrochlorobenzene (DNCB, supplied by MB) at 0.1% in AOO (or suitable vehicle) may be added at the Sponsor's option, especially if optional immunophenotyping endpoints are to be added to the study. These chemicals have produced consistent responses in the LLNA with the solvents AOO or D_3AE 433. Other positive, negative, naïve, or irritant controls may be added by the Sponsor, in consultation with the Study Director (see section 13.1.2).

5.5: Negative Control: There is no suggested negative control for the LLNA. A negative control substance or a naïve control group may be added as an additional group at the option of the Sponsor and at additional expense (see section 13.1.2). All test article groups will be compared to their respective vehicle control group.

5.6: Test Solution Preparation:

5.6.1: Safety: Safety glasses and gloves must be worn during solution preparation. If the test substance, vehicle and/or control are known to present an inhalation hazard, all procedures must be carried out in a fume hood.

5.6.2: Test Article Preparation: The sample preparation will be documented in the raw data. Fresh test substance solutions/suspensions will be prepared on each treatment day. Substances of low solubility can be mixed using a mechanical agitator or using a magnetic stirrer. Heat above 38°C will not be used unless the substance is known to be heat stable.

5.6.3: Test Article Concentrations: The test article is normally assayed at three to five consecutive concentrations from within the following range:

100%, 50%, 25%, 10%, 5%, 2.5%, 1.0%, 0.5%, 0.25%, etc. (w/v for solids, v/v for liquids)

The Sponsor will indicate concentrations to be used in Section 13.4.2 based upon previous experience or studies (if available), structure activity analysis, dermal irritation and solubility. Optimal test concentrations will be prepared based upon the maximum solubility of the test article in the vehicle, while avoiding overt or severe systemic toxicity or local irritation. In the event of no such support data, a Quantitative Irritation Test (see 5.6.3.2) may be required in place of or in addition to an Irritation Prescreen (see 5.6.3.1) to determine irritation and solubility thresholds.

5.6.3.1: Irritation Prescreen: An initial irritation test (ear swelling; edema) will be performed using 100%, 50% and 25% of test article (or the 3 highest concentrations obtainable in chosen vehicle). Six mice (two per concentration) will be used and the prescreen will be conducted under identical conditions as the main study, except for the assessment of lymph node proliferative activity. If no irritation is observed (ear swelling <25%), then these concentrations will be used in the main study. If significant irritation (≥25% increase in ear swelling) is observed, a Quantitative Irritation Test (see 5.6.3.2) should be performed.

5.6.3.2: Quantitative Irritation Test: If irritation (ear swelling; edema) is encountered in the Irritation Prescreen (5.6.3.1), an expanded Quantitative Irritation Test should be performed using 12 additional mice (either 4 test article concentrations at n = 3 mice, or 6 test article concentrations at n = 2 mice); see section 13.1.2. If all doses tested are irritating, additional irritation tests at decreasing concentrations of test article should be performed until the irritation threshold (maximum non-irritating dose) is determined.

5.7: Topical Application:

5.7.1: Safety: Gloves must be worn during this operation.

5.7.2: Application: Each group of mice will be treated by topical application of a different selected concentration of the test substance to the dorsum of both ears one time per day for three consecutive days. Control mice will be treated with the vehicle alone. The application volume (25 µl per ear) will be administered using a positive displacement pipette and will be spread over the entire dorsal surface of the ear. The time of dosing will be recorded.

TSCA SANITIZED

Appendix L
MB 07-15961.26

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

5.8: Administration and Incorporation of BrdU in vivo: Five days after the first topical application of test article, all mice will be injected intraperitoneally with 200 µl of a 5-Bromo-2'-deoxy-Uridine solution (BrdU, 15 mg/ml in PBS).

5.9: Observations:

5.9.1: Dermal Reactions: All mice will be observed once daily for signs of local irritation at the application site.

5.9.2: Systemic: At a minimum, mice will be observed once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations will be systematically recorded, with records maintained for each individual mouse.

5.9.3: Body Weights: Body weights will be recorded pre-test and prior to BrdU injection. Significant weight loss[1] (2 g or more) will be noted and addressed.

5.9.4: Ear Swelling: Both ears of each animal will be observed for edema and/or erythema, and ear thickness measurements will be taken on Day 1 (pre-dose), Day 3 (at approximately 48 hours after the first dose), and on Day 6, using a thickness gauge (digital micrometer or Peacock Dial thickness gauge).

5.10: Post Mortem Lymph Node Extraction:

5.10.1: Sacrifice: Animals showing severe and enduring signs of distress and pain, or animals in a moribund condition and not expected to survive until the next observation interval will be humanely sacrificed using CO_2. On the day of study termination, five (5) hours after the injection of BrdU, the surviving mice will be euthanized by asphyxiation with CO_2.

5.10.2: Excision and Preparation of Lymph Node Cells: Following sacrifice, all of the draining auricular lymph nodes from each mouse will be excised and combined. On an individual animal basis, single cell suspensions of lymph node cells (LNC) will be prepared from the collected lymph nodes by gentle disaggregation, and erythrocytes will be lysed and removed from the LNC suspension.

5.11: Optional Immunophenotyping Cell Treatment: An aliquot of the cells will be stored at 4°C in storage media for up to 72 hours for optional flow cytometry analysis of immunophenotype or surface marker expression, e.g., %B220+, %CD3+, %CD69+, and/or %I-A^k+ cells, as per MB SOP vol. VII.D.1.

5.12: Fixation of Cells: An aliquot of the LNC will be preserved with a suitable buffer or alcohol and will be stored at -18 to -30°C until further processed. Storage should not exceed two months.

5.13: Determination of Cell Proliferation:

5.13.1: Enumeration of Cells: After propidium iodide staining, a single-cell suspension of LNC will be analyzed using a Becton-Dickinson flow cytometer specifically programmed for propidium iodide staining to enumerate nucleated cells in the lymph node cell suspension. The total number of cells per mouse will be calculated from the values obtained by multiplying by the appropriate dilution factors.

[1] Page updated 03/21/07 to clarify significant body weight changes

TSCA SANITIZED

Appendix L
MB 07-15961.26

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

5.14: Determination of BrdU Incorporation:

5.14.1: Acid Denaturation of DNA: DNA of LNC will be acid denatured so that the BrdU antibody can access and quantitatively interact with the BrdU that has been incorporated into the cellular DNA.

5.14.2: Neutralization of the Cellular Material: Samples will be neutralized by washing the cells with borate buffer (pH 8.5), or other comparable neutralization buffer.

5.14.3: BrdU-specific Staining of Cells: Nuclei will be washed with a staining buffer and incubated with BrdU-specific fluorescent antibody conjugate. The nuclei will be washed again with staining buffer and resuspended in PBS containing RNase A and the DNA-specific dye propidium iodide. Following at least a 30-minute incubation at room temperature, the total DNA content of the nuclei, as well as the percentage of nuclei staining positive for BrdU (i.e. percentage of proliferating lymphocytes), will be determined using flow cytometry.

5.15: Test Duration: The test duration of the study is at least eleven (11) days.

6.0: FLOW CYTOMETRY:

6.1: Flow Cytometer: Flow Cytometry and all cell processing will be conducted according to MB Research Laboratories Standard Operating Procedures. Lymph node cell analyses will be performed using a Becton-Dickinson FACScan flow cytometer using 15 mW of power at 488 nm excitation wavelength. BD CellQuest ver. 3.3 acquisition software on a Macintosh G4 acquisition system will be used to capture and store data (List Mode Data files, as .LMD or .FCS) on a dedicated secure network drive. Data files will be analyzed using FlowJo for PC or CellQuest to determine appropriate analysis gate and % positive LNC populations.

7.0: DATA ANALYSIS AND CALCULATION OF STIMULATION INDEX (SI):

7.1: Data Analysis: For analysis of individual animal lymph node sets (right and left side draining local nodes), the proliferative response of lymph node cells (LNC) will be expressed as the total number of BrdU-positive lymphocytes per (individual animal) lymph node sets. The mean value of the total number of BrdU-positive cells and its associated standard deviation (S.D.) will be calculated for each group. The SI, i.e. the ratio of the mean BrdU incorporation into LNC of each test article group divided by that of the vehicle group, will be calculated for each test group according to Equation 1 below:

Equation 1:

$$SI = \frac{\text{Mean \#BrdU+ cells in Treatment Group}}{\text{Mean \#BrdU+ cells in Vehicle Group}}$$

7.2: Equivocal Results: In the case where dose-related increases in cell proliferation (i.e., BrdU-positive cells) result in a SI that approaches but does not reach 3, the regulatory guidelines may require that additional tests be performed using higher concentrations of the test substance (or in another vehicle) if possible. In such cases, the effect of the vehicle on the outcome should also be examined.

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Appendix L
MB 07-15961.26

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

7.3: Statistical Analysis: For each test group, the individual SI values along with the mean SI and standard deviation will be calculated. If further statistical analysis is required, the analysis will be performed only upon request by the sponsor.

8.0: DATA INTERPRETATION:

8.1: Interpretation: A substance will be regarded as a sensitizer in the LLNA if at least one concentration of the test article results in a 3-fold or greater increase in LNC proliferation relative to that of Control (Vehicle) lymph nodes, as indicated by an SI ≥3.0. The data should also be compatible with a biological dose response, although an allowance must be made, especially at high topical application concentrations, for local irritation, systemic toxicity or immunological suppression.

9.0: PROCESSING OF TISSUE:

9.1: Tissues: Other than the lymph nodes processed as above, no other tissues will be taken.

9.2: Optional Tissues: At the option of the Sponsor, the dosage site (ears) will be excised and preserved in 10% neutral formalin for H&E staining and histopathological evaluation at an additional cost. Other tissues or organs may be specified by the Sponsor to be isolated and preserved. Histopathology will be performed by W. Ray Brown, D.V.M., Ph.D., DACVP, Research Pathology Services, Inc., New Britain, PA.

10.0: REVISION OF THE PROTOCOL: Any amendment to or deviation from this protocol will be fully documented in the study file, including the reason for the change, the authority for said change and the date thereof.

11.0: RECORDS TO BE MAINTAINED:

11.1: Collection of Data: All data generated during the conduct of the study will be recorded in ink on worksheets. All entries will be dated, initialed and verified by another person. Flow cytometry data files will be write-protected, backed-up and a copy stored off-site. The original computer-acquired data will be analyzed and histograms, dot plots and % positive LNC will be printed out for each animal and endpoint, and included with the raw data.

11.2: Reports:

11.2.1: Draft Report: A draft report will be submitted to the sponsor prior to submission of the final report.

TSCA SANITIZED

Appendix L
MB 07-15961.26

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)

11.2.2: Final Report: Following approval by the sponsor of the draft report, the final report will be submitted and will include, but not be limited to:

- Species, strain, sex, number, age and source of test animals
- Equilibration, housing conditions during exposure and post-exposure, bedding material, room temperature and humidity, light/dark cycle, diet and water
- Method of random assignment
- Physical nature, purity, stability, and lot number of test article
- Justification for choice of solvent/vehicle
- Individual and test group data (i.e., mean and std. dev.) presented in tabular form
- Systemic signs and body weights for each group
- Description of adverse effects of treatment on the mice
- List of references cited in the report, including references to any published literature used in developing the protocol, performing the testing, making and interpreting observations and compiling and evaluating results
- The number of lymphocytes, the %BrdU+ cells and the #BrdU+ cells for each animal will be determined. The calculated Stimulation Index for each group (compared to its respective Vehicle Control group) will be presented in tabular form

11.3: Retention of Data:

11.3.1: Raw Data: The original raw data will be filed at MB Research by project number.

11.3.2: Final Report: The original final report will be submitted to the sponsor and a true and certified copy of the original report will be filed at MB Research by sponsor name and MB project number.

11.3.3: Test Article: Any remaining test article will be returned to the sponsor upon submission of the study report.

11.3.4: Tissues, cells, blocks & slides will be stored at MB Research and indexed by sponsor name and MB project number. The sponsor will be contacted to determine final disposition upon submission of the report.

12.0 GOOD LABORATORY PRACTICES:

12.1: This study will be conducted in accordance with the Good Laboratory Practices of the EPA, 40 CFR 160 and 792, FDA 21 CFR 58, and as specified in the Principles on Good Laboratory Practice, published by the Organization for Economic Cooperation & Development (OECD), 1997.

12.2: Protocol: MB Research will have on file a copy of this protocol, signed and dated by both the responsible MB Study Director and the Sponsor's authorized representative.

12.3: Quality Assurance: The Quality Assurance Unit will inspect at least one critical phase of this study, audit the raw data and audit the report in accordance with the Standard Operating Procedures of MB and the applicable government regulations.

TSCA SANITIZED

Appendix L
MB 07-15961 26

STUDY TITLE: Local Lymph Node Assay in Mice (LLNA)	MB RESEARCH LABS
Draft Report: ☒ Yes ☐ No	PROTOCOL NO: 5650M
Reports and Invoices to be sent to: Melinda Mitchell	PAGE NO : -10 of 11-

13.0 SPONSOR REQUEST:

* 13.1: This Sponsor requests that this protocol be implemented:
☐ As written (or) ☒ Modifications per attached

13.1.1: Options:
Other Vehicle (see sections 3.5 & 5.3.2): _____

13.1.2: Options (at additional cost):
Quantitative Irritation Test conc's: ____% ____% ____% ____% ____% ____%
Additional Vehicle (see sections 3.5 & 5.3.2): _____
Additional Controls (see 5.4 & 5.5): ☐ 25% SLS & DMSO ☐ Naive ☐ Other: _____
Immunophenotyping: ☒ %B cells ☒ %T cells ☐ %I-A^b+ cells ☐ %CD69+ cells
Optional additional Tissues (e.g., ears-see 9.1): _____

13.2: Will report be submitted to a regulatory agency? ☐ No ☒ Yes (agency): EU, EPA

13.3: 3M Study Identification will be indicated in the report and supporting documentation as: 07-106

13.4: Test Article will be identified in the report and supporting documentation exactly as indicated below.

13.4.1: Identity: The test article is identified as follows: MTDID 6695

pH (when applicable) 6-8 Lot/Batch #: _____ CAS #: _____

13.4.2: Test Concentrations (%v/v or %w/v): ____% ____% ____% Additional concs: _____

13.4.3: Characterization of the test article is required in support of data submissions and should include identity, strength, purity, composition, stability and uniformity. This data must be reviewed by the Study Director prior to study initiation and included in the final report. (EPA 40 CFR 160.105 and 792.105; FDA 21 CFR 58.105, OECD 6.2). This information is:
☒ provided (C of A) (or) ☐ not available

13.4.4: Material Safety Data Sheet Supplied: ☒ Yes ☐ No

13.4.5: DOT Hazardous Material: ☒ No ☐ Yes (indicate DOT shipping Name) _____
EPA Hazardous Waste: ☒ No ☐ Yes (indicate EPA Waste Number) _____

13.4.6: Return Residual Test Article: (indicate name, address and phone #): 3M Center
St. Paul, MN 55144-1000

Shipping Instructions: (Call or refer to Study Initiation Information for costs)
☒ UPS / Ambient temperature (no charge) Maintain above freezing. ☐ Express carrier / Ambient temperature
☐ Overnight carrier / Dry ice ☐ Overnight carrier / Ice packs

13.5: Authorization Statement: This protocol is authorized for implementation at MB Research. This study is necessary in order to estimate any potential adverse effects that may be encountered through use of the test compound. To the best of my knowledge and information, this test is not an unnecessary duplication of any previous studies.

13.5.1: Confidentiality: Study results and reports will be released only to those individuals identified in sponsor's letter of March 10, 2006 signed by James P. Zappia, Manager, Strategic Toxicology.

BY: Steven C Gordon 6/15/2007
(signature) (date)
Steven C. Gordon, PhD, DABT
(type/print name)
Senior Toxicology Specialist
(title)
scgordon@mmm.com
(email)

FOR: 3M Corporate Toxicology & Regulatory Services
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1755 woratz road, p.o. box 178
phone: (215) 536-4110
spinnerstown, pa 18968
fax: (215) 536-1816

* No modifications per sponsor email 7/12/07 TF 7/13/07

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Appendix L
MB 07-15961.26

STUDY TITLE: Local Lymph Node Assay In Mice (LLNA)

MB RESEARCH LABS
PROTOCOL NO: 5650M
PAGE NO: 11 of 11

14.0 MB RESEARCH ACKNOWLEDGMENT: Request for implementation of this protocol and receipt of the test article is acknowledged by MB Research.

14.1 Test Article Identity: MTD1D 6675

14.1.1: Date Received: 7/6/07

14.1.2: Physical Description: clear liquid

14.1.3: Test Article Characterization:

14.1.3.1: ☐ Not supplied by Sponsor, or

14.1.3.2: ☒ Received and Reviewed by Study Director.

14.2: MB Project Number assigned to this study: 07-15961.26

14.3: Animal Supplier: The Licensed USDA animal supplier is: Jackson

14.4: Proposed Study Dates:

14.4.1: Experimental Start Date: 7-18-07

14.4.2: Experimental Term Date: 8-16-07

14.4.3: Study Completion Date (Submission of Report): Approximately 6-8 weeks following Experimental Term Date.

14.5: Approval: There are currently no suitable non-animal alternatives to this study as determined according to MB Research SOP Vol. III A. This protocol is designed to avoid or minimize discomfort. The procedures will be performed by personnel thoroughly trained in the humane care and use of laboratory animals. If pain does occur as a result of the nature of the test article being used, it will be addressed according to MB SOP Vol. III A. This protocol is approved for implementation at MB Research by the below named MB Study Director.

Melissa Veale 7-18-07
by: date
Study Director
Testing Facility: MB Research Laboratories
1755 Wentz Road, P. O. Box 178
Spinnerstown, PA 18968

This protocol was originally reviewed by the Institutional Animal Care and Use Committee (IACUC) of MB Research on the date indicated below and found to comply with acceptable standards of animal welfare and humane care. The IACUC committee will review this protocol on an annual basis. This review will be documented in the IACUC minutes and included in the semi-annual report to the institutional official.

DATE: 04/01/1999