Appendix C

Comparison of LLNA Responses for Substances Tested in CBA and BALB/C Mice

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1.0 Introduction

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the LLNA is a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many types of substances (Haneke, et al., 2001). The LLNA provides several advantages compared to guinea pig methods, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information (Dean, et al. 2001; Sailstad et al., 2001). The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel assessment of LLNA validation status (ICCVAM 1999).

The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (OECD 2002; ISO 2002; EPA 2003) and is now commonly used worldwide. The recently updated ICCVAM-recommended LLNA protocol states that mouse strains other than CBA may be used in the LLNA if it is sufficiently demonstrated that these animals perform as well as CBA mice in the LLNA (ICCVAM 2009).

Although CBA/J and CBA/Ca mice are currently recommended as the preferred mouse strains in national and international LLNA test guidelines (OECD 2002; EPA 2003), the LLNA was originally developed using BALB/c mice (Kimber et al. 1986). Kimber and Weisenberger (1989) observed that *in vitro* proliferation of lymph node cells in response to exposure to 2,4-dinitrochlorobenzene was stronger in CBA/Ca mice than in BALB/c, and chose to focus on using CBA/Ca mice in further development efforts for the LLNA.

Woolhiser and co-workers assessed LLNA responses in various mouse strains including CBA and BALB/c. They found essentially equal levels of lymph node proliferation (as measured by incorporation of 3H-thymidine into the draining auricular lymph nodes) in both strains following exposure to the sensitizers α-hexylcinnamaldehyde (HCA), 2,4-dinitrofluorobenzene (DNFB) and toluene diisocyanate (Woolhiser et al., 2000). Other U.S. groups have also published LLNA studies using BALB/c mice, including the National Institute for Occupational Safety and Health, the Dow Chemical Corporation, and the National Toxicology Program (Anderson et al. 2009; Boverhof et al. 2009; NTP 2005) and continue to use them today.

In order to further evaluate the impact of using different strains and substrains of mice in the LLNA, the study reported here is a retrospective evaluation of the performance of the LLNA in studies using CBA mice with studies using BALB/c mice. LLNA results are compared from studies done with CBA and BALB/c mice using the same test substances in the same vehicles.

2.0 Methodology

The information summarized here is based on LLNA data derived from a database of over 600 substances tested in the LLNA. Data were extracted from published reports or submissions in response to a *Federal Register* (*FR*) notice requesting LLNA, guinea pig, and/or human skin sensitization data and experience (Vol. 72, No. 95, pp. 27815-27817[1]). Key words used in the online searches for this evaluation were "LLNA" OR "Local Lymph Node" OR "Local lymph node" OR "local lymph node". Papers that contained studies on BALB/c were identified by appending AND "balb/c" to this search string. Forty-one such papers identified by the AND "balb/c" search were examined for BALB/c data appropriate for inclusion in this study.

The primary consideration for inclusion of data from published studies was the identification of test substances for which LLNA studies in the same vehicle existed. In general, published studies that

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[1] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

were included in this evaluation followed the LLNA protocol in the Organisation for Economic Co-operation and Development (OECD) Test Guideline 429 (OECD 2002). However, some exceptions were made since many of the published BALB/c studies were done prior to the formal adoption of TG 429. Exceptions to the OECD protocol include studies in which lymph nodes were harvested on days 3, 4, 5, and 6 after study initiation, as well as studies that used 2 or 3 mice per treatment group. Studies that included other modifications (e.g., pretreatment of mice with sodium lauryl sulfate before application of the test substance) were excluded. The complete database is in **Annex I.**

An LLNA result was identified as positive if an SI value ≥ 3.0 occurred at any concentration tested. Overall LLNA outcomes for individual substances were made according to the most prevalent outcome, or on a most conservative basis if an equal number of positive and negative studies were found (i.e., considered positive). Since this was a retrospective study, there were substances with multiple studies using the same strain. For each such substance, LLNA outcome was based on the most prevalent study result (positive vs. negative), or considered positive if an equal number of positive and negative studies were found. EC3 values (the concentration of a test substance necessary to cause an SI value of 3) were calculated according to the methods used by Ryan and co-workers (Ryan et al., 2007). In the event that an EC3 value could not be calculated using these methods due to an inadequate dose response, the study was still designated as either positive or negative for the purpose of calculating agreement between strains, based on the decision criterion of SI> 3 as the basis for a positive.

3.0 Results

3.1 Characteristics of the Database

A summary of the responses in LLNA studies conducted with CBA and BALB/c mice is shown in **Table C-1.**

Table C-1 Summary of LLNA Responses from CBA and BALB/c

Test Substance	Vehicle	No. of Studies								
		All Strains	CBA			BALBc			Avg EC3 (%)	
		Total	Total	Pos	Neg	Total	Pos	Neg	CBA	BALBc
3-Amino-5-mercapto-1,2,4-triazole	DMSO	2	1	1	0	1	1	0	11.6	5.2
Benzocaine	AOO	5	4	1	3	1	0	1	NC	NC
Cobalt chloride	DMSO	3	2	2	0	1	0	1	0.6	NC
2,4-DNCB	AOO	14	10	10	0	4	4	0	0.052	0.116
2,4-DNFB	AOO	3	1	1	0	2	2	0	0.016	0.024
Eugenol	AOO	9	8	8	0	1	1	0	14.3	13.8
Eugenol	ACE	2	1	1	0	1	0	1	18.2	NC
Formaldehyde	DMF	2	1	1	0	1	1	0	0.27	0.11
Glutaraldehyd	DMF	2	1	1	0	1	1	0	0.07	0.09

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HCA	ACE	5	4	4	0	1	1	0	5.8	12.9
Isoeugenol	AOO	33	32	32	0	1	1	0	1.4	0.8

continued

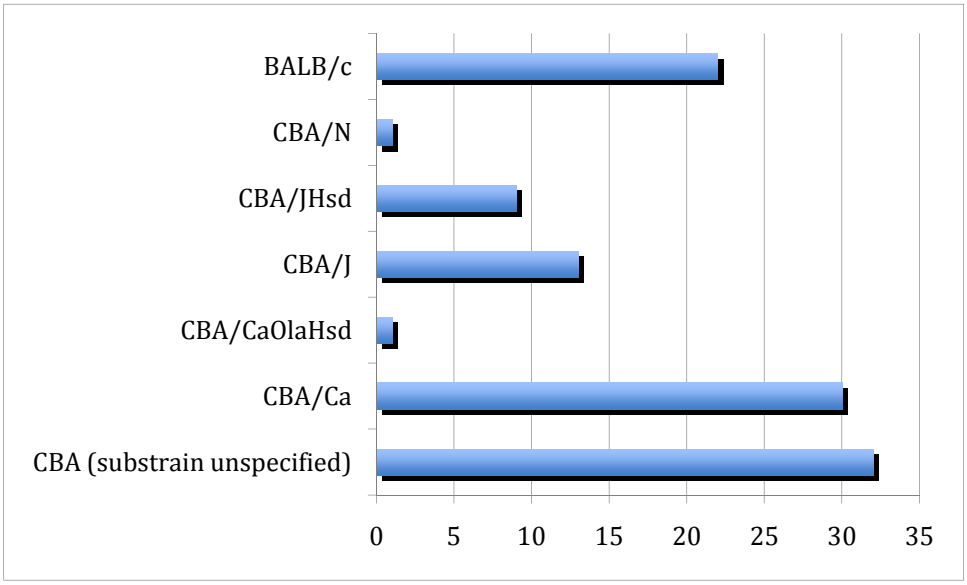
Table C-1 Summary of LLNA Responses from CBA and BALB/c (continued)

Test Substance	Vehicle	No. of Studies								
		All Strains	CBA			BALBc			Avg EC3 (%)	
		Total	Total	Pos	Neg	Total	Pos	Neg	CBA	BALBc
Methyl salicylate	AOO	7	6	0	6	1	0	1	NC	NC
Nickel sulfate	DMSO	2	1	1	0	1	0	1	1.5	NC
Oxazolone	AOO	6	5	5	0	1	1	0	0.0018	IDR
Potassium dichromate	DMSO	10	8	8	0	2	1	1	0.09	0.2
Trimellitic anhydride	AOO	3	1	1	0	2	2	0	9.2	0.15
Total No. Studies		108	86	77	9	22	16	6		

Abbreviations: ACE = acetone; AOO = acetone/olive oil; DMF = dimethylformamide; DMSO = dimethylsulfoxide; DNCB = dinitrochlorobenzene; DNFB = dinitroflurobenzene; EC3 = estimated concentration needed to produce a stimulation index of 3; HCA = α-hexylcinnamic aldehyde; IDR = Inadequate dose response to calculate an EC3 value; LLNA = local lymph node assay; N = No; NC = not calculated; Neg = negative; Pos = positive.

The database evaluated contains results from a total of 108 independent LLNA studies, representing 16 different test substances; 86 of the studies were done with CBA and 22 with BALB/c. Substrains of CBA mice used in the studies were not always specified; specified CBA substrains included CBA/Ca, CBA/CaHsd, CBA/J, CBA/JHsd and CBA/N. None of the studies using BALB/c mice specified a substrain. **Figure C-1** shows a frequency distribution of the substrains used in the studies analyzed. The substrain used in a particular study and the supplier (if known) is indicated for each study in **Annex 1**.

Figure C-1 Substrain Frequency Distribution



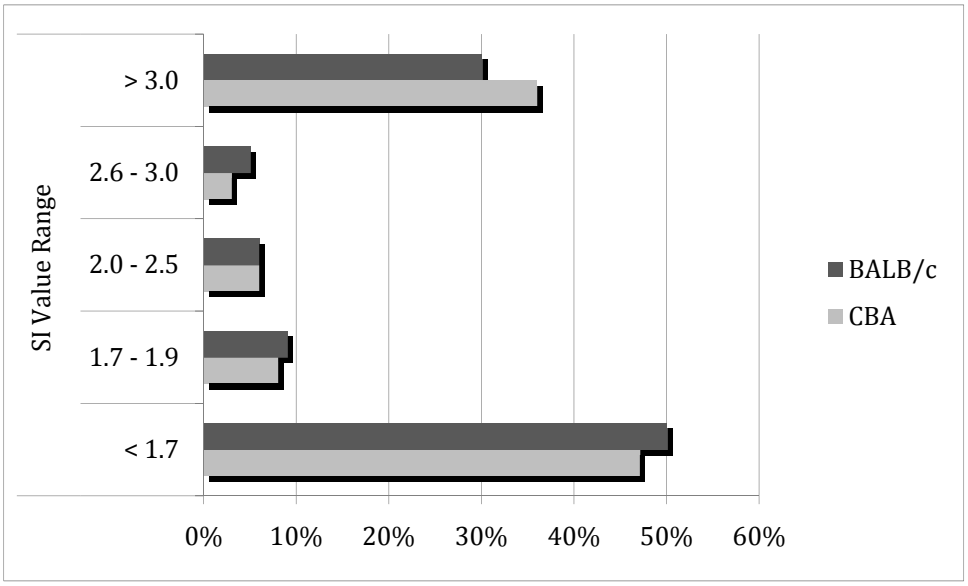
Four different vehicles were represented, with acetone-olive oil (AOO, 80 studies) being the most prevalent, followed by dimethylsulfoxide (DMSO, 17 studies), acetone (ACE, 5 studies) and dimethylformamide (DMF, 4 studies). Only one nonsensitizer (as classified by results in guinea pigs and humans), methyl salicylate, was included. The EC3 values for the 15 sensitizers (as determined from CBA LLNA data) included in the database ranged from 0.0018% (for oxazolone in AOO) to 18.2% (for eugenol in ACE) (**Table C-1**).

Current ICCVAM-recommended LLNA performance standards (ICCVAM 2009) recommend that EC3 values for HCA and DNCB determined in different laboratories should fall into a range of 0.5-2.0x of a reference value; in this study, 29% of the EC3 values for all sensitizers determined in BALB/c fall within this range, if the EC3 value determined in CBA is used as the reference. Neither the EC3 value determined in BALBc for DNCB, or for HCA, falls within this range (**Table C-1**). However, it should be noted that most of the EC3 values determined in both strains were based on a very limited number of studies; for CBA, 8/16 EC3 values were based on one or two LLNA studies, for BALB/c, 13/16 EC3 values were based on one or two LLNA studies. No EC3 value for oxazolone was determined in BALB/c because the dose response data were inadequate to do so.

3.2 Comparison of Responses in the LLNA from CBA and BALB/c Databases

Initially, results from LLNA studies using CBA mice (75 substances, 83 LLNA studies) were compared to results from LLNA studies using BALB/c mice (39 substances, 41 LLNA studies) (ICCVAM 2009). The percentage of positive LLNA studies (i.e., SI $\geq$ 3.0) using either CBA (59% [49/83]) or BALB/c (63% [26/41] mice were similar. **Figure C-2** shows the frequency distribution of LLNA responses from 277 test substance doses that fall into the indicated ranges of SI values. However, this does not include a comparison of results from the same substances tested in the same vehicles. The study described in this report was done to compare results of substances tested in the same vehicle in both CBA and BALB/c.

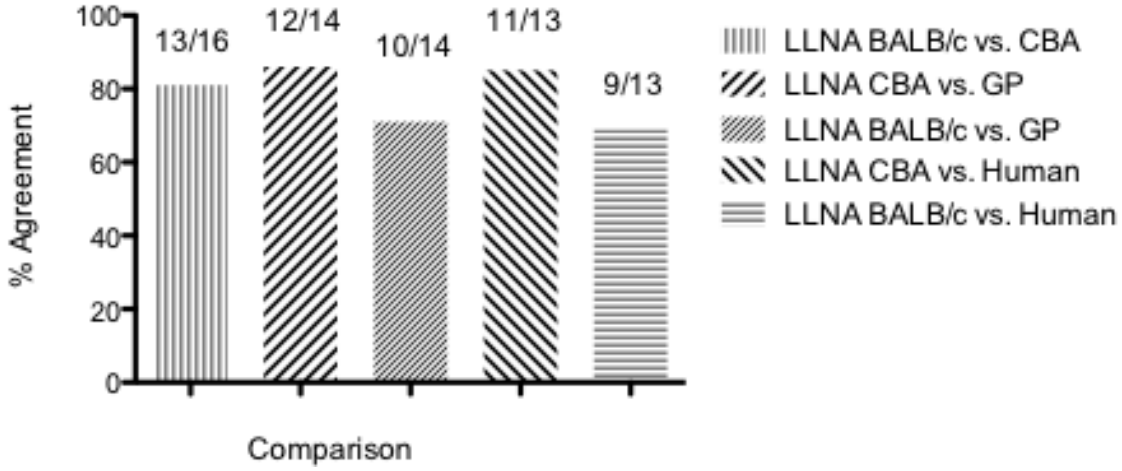
Figure C-2 Comparison of LLNA Responses from CBA and BALB/c Databases (ICCVAM 2009)



Abbreviation: No. = number; SI = stimulation index

The database analyzed here contains data for 16 substances for which there is LLNA data for both CBA and BALB/c in the same vehicle. Thirteen of these substances had GP reference data and 12 had human reference data. Two substances, 3-Amino-5-mercapto-1,2,4-triazole and 2,4-dinitrofluorobenzene, had neither GP nor human reference data; and one substance, trimellitic anhydride, had GP reference data but no human reference data. For this database, 92% (12/13) of the substances were classified as sensitizers in the GP, 92% (11/12) of the substances were classified as sensitizers in humans, 8% (1/13) were classified as nonsensitizers in the GP and 8% (1/12) were classified as nonsensitizers in humans. **Figure C-3** provides a comparison of the performance of the LLNA when the two strains are compared to each other, and to GP and human outcomes.

Figure C-3 Comparison of the Performance of the LLNA using CBA or BALB/c Mice



Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = local lymph node assay; No. = number.

GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test. Human refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

LLNA outcomes using BALB/c are in agreement with LLNA outcomes obtained with CBA for 81% (13/16) of the test substances. LLNA outcomes with CBA agree with GP outcomes for 86% (12/14) of the test substances and with human outcomes for 85% (11/13) of the test substances; in contrast, LLNA outcomes with BALB/c agree with GP outcomes for 71% (10/14) of the test substances and with human outcomes for 69% (9/13) of the test substances.

Table C-2 contains LLNA data for three substances (cobalt chloride, nickel sulfate, and eugenol) for which the overall LLNA results were different between CBA and BALB/c, or between one of the mouse strains and guinea pig or human reference data. In the LLNA studies for cobalt chloride and nickel sulfate considered in this investigation, the LLNA results using CBA were concordant with guinea pig and human reference tests, while those using BALB/c were discordant. However, the discordant results obtained in BALB/c were based on a single study for each metal compound. The negative study for nickel sulfate using BALB/c was a 4-day study, while the positive study in CBA was a 6-day study. Furthermore, the LLNA response was a borderline positive in CBA (maximum SI=3.1), and the maximum SI for BALB/c mice was SI=2.46; **Table C-2**). For these reasons there is insufficient information to draw conclusions about the LLNA response to metals in BALB/c. It should also be noted that metal compounds (ICCVAM 1999) are known to produce variable LLNA responses in CBA.

Table C-2 Substances Discordant Between the LLNA, GP, and Human

Chemical Name	LLNA Vehicle	Conc. (%)	SI	EC3 (%)	Mouse Strain	LLNA Call	LLNA Study Length (Days)	Overall LLNA Call[2] (CBA)	Overall LLNA Call[2] (BALB/c)	Overall GP[1] Call[2]	Overall Human[3] Call[2]	LLNA Ref	GP Ref	Human Ref
Eugenol	ACE	25, 50, 75	5.4, 10.6, 10.5	18.5	CBA/J	+	5	+	-	+	+	Gerberick et al. (1992)	Basketter et al. (1999)	Basketter et al. (1999)
		10, 20	1.07, 1.89	NC	BALB/c	-	4					Sailstad et al., (1995)		
Cobalt chloride	DMSO	0.5, 1.0, 2.5	3.2, 3.7, 2.8	0.4	CBA/Ca	+	5	+	-	+	+	Basketter and Scholes (1992)	Basketter et al. (1999)	Kligman (1966)
		0.5, 1.0, 2.5, 5.0	2.1, 3.5,3.8, 7.2	0.8	CBA/N	+	4					Ikarashi (1992b)		
		1.0, 2.5, 5.0	1.5, 1.6, 2,7	NC	BALB/c	-	4					Manderve lt et al. (1997)		
Nickel sulfate	DMSO	0.25, 0.5, 1, 2.5, 5	1.3, 1.4, 1,4, 1.8, 3.1	4.8	CBA/J	+	6	+	-	+	+	Ryan et al. (2002)	Basketter and Scholes (1992)	Kligman (1966)
		2.5, 5,	2.19, 2.46	NC	BALB/c	-	4					Ikarashi et al, (1992a)		

Abbreviations:

AOO = acetone/olive oil; Conc. = concentration; DMSO=dimethylsulfoxide; EC3 = estimated concentration needed to produce a stimulation index of 3; GP = guinea pig; Ind. Conc. = induction concentration; LLNA = local lymph node assay; NC = not calculated since SI<3.0; SI = stimulation index; Veh. = vehicle

[1] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[2] Human refers to outcomes obtained by studies conducted using either the human repeat insult patch test or the human maximization test, or inclusion in a human patch test allergen kit.

[3] (-) = nonsensitizer, (+) = sensitizer

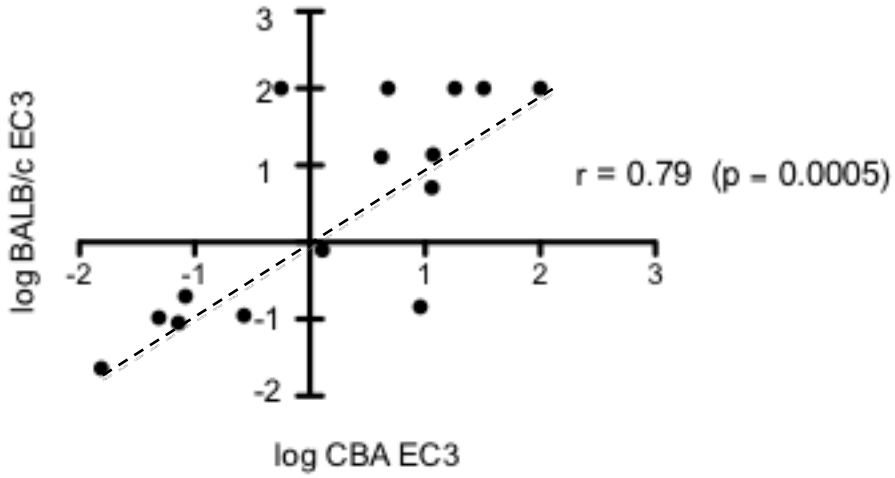
In the LLNA studies for eugenol with acetone as the vehicle, the LLNA results using CBA were concordant with guinea pig and human reference tests, while those using BALB/c were discordant. The differences between CBA and BALB/c studies may be due the large differences in the concentration ranges used, where the maximum concentration used in the CBA study was almost 4-fold higher than that used in the BALB/c study. It should also be noted that BALB/c and CBA studies for eugenol in which AOO was used as the vehicle were both positive. (**Annex 1**).

3.3 Correlation of EC3 Values Obtained with CBA and BALB/c Mice

A correlation analysis between EC3 values calculated using LLNA data from each of the two strains was done. If there were multiple LLNA studies for a strain, a geometric mean EC3 value was used in the correlation analysis. Since the EC3 values for the test substances in this analysis spanned six orders of magnitude (range = 0.0018% to 100%), the mean EC3 values were log transformed prior to analysis. Oxazolone was not included in this analysis because the dose response obtained with BALB/c mice was inadequate to allow calculation of an EC3 value (**Table C-1**).

Spearman's rank correlation is used for rating the extent of agreement with the 'true" ranking of a set of observations (Steel and Torrie, 1980). In this analysis, the CBA EC3 results were considered the "true" ranking. A highly significant ($p \leq 0.0005$) positive correlation ($r = 0.79$) was obtained between EC3 values calculated from LLNA studies in both strains (**Figure C-4**).

Figure C-4 Correlation of EC3 Values Obtained with CBA and BALB/c Mice



Log-transformed geometric mean EC3 values for 15 of the 16 substance-vehicle groups shown in **Table 2**. r = Spearman's Rank correlation coefficient.

NOTE: An EC3 value of 100% was assigned to negative LLNA results in order to exceed all positive values, so that they could be included in the correlation analysis.

Among the 10 substances for which an EC3 was calculated in both CBA and BALC/c studies, 5/10 were lower CBA and 5/10 were lower in BALB/c. (**Table C-1**).

As stated previously, it should be noted that most of the EC3 values determined in both strains were based on a very limited number of studies; for CBA, 50% (8/16) EC3 values were based on one or two LLNA studies, and for BALB/c, 81% (13/16) EC3 values were based on one or two LLNA studies (**Table C-1)**.

3.4 Conclusions

This study complements a previous study (ICCVAM 2009), which concluded that the percentage of positive LLNA responses study were the same between studies with CBA or BALB/c mice. However, there was no substance-by-substance comparison (i.e., the respective databases were compared *in toto*, regardless of test substance or vehicle). Therefore, the present study compares results from LLNA studies with CBA and BALB/c mice using the same test substances in the same vehicles.

Current testing guidelines (OECD 2002; EPA 2003) recommend using CBA mice unless it is sufficiently demonstrated that significant strain-specific differences in the LLNA response do not exist. When compared to LLNA studies using CBA mice (the strain specified in the ICCVAM-recommended LLNA protocol [ICCVAM 2009]), results of studies done on the same substances in BALB/c were in agreement most of the time (81% [13/16])
(**Figure C-3**). Also, there was a positive rank correlation ($r = 0.79$) between EC3 values ($p \leq 0.0005$) (**Figure C-4**). Where there were different outcomes (n=3) between the two mouse strains, the CBA studies were positive (which was also concordant with the human and GP outcomes) while the BALB/c studies were negative (and thereby discordant with the human and GP outcomes) (**Table C-2**).

These results suggest that further characterization of strain and substrain differences in needed. Until such additional information becomes available, caution should be used prior to selecting a mouse strain other than CBA for use in the LLNA for regulatory testing.

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Annex I

Data for Substances Tested in the LLNA in s CBA and BALB/c Mice

List of Abbreviations and Acronyms

ACE	acetone
AOO	acetone: olive oil (4:1)
CASRN	Chemical Abstract Services Registry Number
Conc.	concentration
DMF	N, N-dimethyl formamide
DMSO	dimethyl sulfoxide
EC3	estimated concentration needed to produce a stimulation index of 3
GP	guinea pig
LLNA	murine local lymph node assay
MEK	methyl ethyl ketone
NA	not available
Veh.	Vehicle
SI	Stimulation index
+	Sensitizer
-	Non-sensitizer