



Allergic Contact Dermatitis (ACD) Case Studies

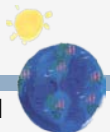
ICCVAM Workshop Series on Best Practices
for Regulatory Safety Testing: Assessing the
Potential for Chemically Induced Allergic
Contact Dermatitis

January 20, 2011

William H. Natcher Conference Center
National Institutes of Health
Bethesda, MD

Case Study 1: Introduction

- You have submitted a protocol to the IACUC to use the LLNA to assess the ACD hazard potential of Chemical A
- The IACUC is pleased that you plan to use the LLNA rather than the guinea pig maximization test, the test your lab traditionally uses
- The IACUC further responds that you should consider performing the reduced LLNA (rLLNA) in order to reduce the number of animals used



Case Study 1: Prior Chemical Information

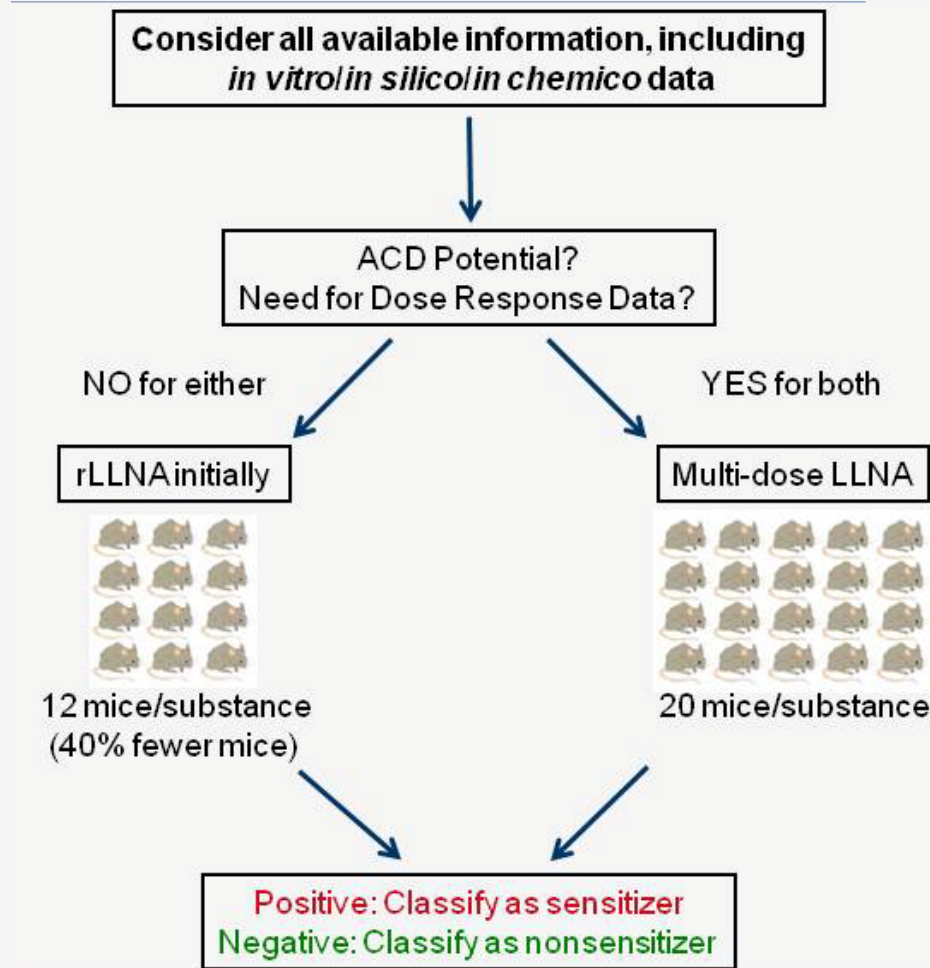
- Information available for Chemical A
 - Molecular weight > 600
 - Log K_{ow} = 2.84
 - No structural alerts for skin sensitization
 - Structurally similar to Chemical B, which is a nonsensitizer
 - No other information is available
- Based on the information above, do you suspect that Chemical A may be a sensitizer or nonsensitizer?
 - Nonsensitizer based on its high molecular weight, similarity to Chemical B, and the lack of structural alerts for skin sensitization
 - Substances with molecular weights > 500 are less likely to be sensitizers due to limited penetration of the stratum corneum¹
 - 70% (12/17) of substances in the NICEATM LLNA database with a molecular weight > 600 were nonsensitizers

¹Bos JD, Meinardi MMHM. 2000. Exp Dermatol 9: 165-169.



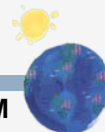
Case Study 1: Decision Strategy for Using rLLNA

- You remember the following decision strategy and revise the protocol to perform the rLLNA



Case Study 1: IACUC Follow-up

- The IACUC was pleased with your revision to use the rLLNA and expeditiously approved the protocol
- Is there sufficient information to determine the dose for testing in the rLLNA or should a prescreen test be performed?
 - The dose tested must be the maximum concentration that does not produce overt systemic toxicity and/or excessive local skin irritation in the mouse
 - All existing toxicological information (i.e., acute toxicity and dermal irritation), structural information, and physicochemical information on Chemical A (and/or Chemical B, a structurally related substance) should be considered
 - A prescreen test must be performed because
 - There is no information on the doses that produce systemic toxicity or local skin irritation for Chemical A
 - The test and dose information for Chemical B is unavailable



Case Study 1: Dose Selection

- You perform a prescreen test using three doses with 2 mice/dose
 - The doses include the maximum soluble dose in acetone: olive oil (4:1) (AOO), 10%, with 5% and 2.5% as the lower doses
 - These doses are applied to the dorsum of the ears on days 1, 2, and 3
 - Body weights are measured on days 1 and 6
 - Ear thickness is measured on days 1, 3, and 6
 - Erythema of the dorsal surface of the ear is scored on days 1, 3, and 6
- No clinical signs of toxicity were observed at any dose; body weight, ear thickness, and ear erythema data are shown on the next slide



Case Study 1: Prescreen Test Data for Chemical A

Chemical A Dose	Animal	Change in Body Weight ^a	Change in Ear Thickness Day 3 ^b	Change in Ear Thickness Day 6 ^b	Erythema Score Day 1 ^c	Erythema Score Day 3 ^c	Erythema Score Day 6 ^c
2.5%	1	+5.6%	10.7%	9.5%	0	0	0
	2	+4.9%	9.8%	10.2%	0	1	0
5%	3	+2.2%	16.9%	16.9%	0	2	1
	4	+3.7%	20.2%	18.9%	0	1	1
10%	5	-6.3%	26.2%	30.1%	0	3	2
	6	-7.1%	35.1%	33.4%	0	3	3

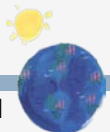
Erythema score: 0 = no erythema; 1 = very slight erythema (barely perceptible); 2 = well-defined erythema; 3 = moderate to severe erythema.

^a Percent difference of Day 6 body weight compared to Day 1 body weight.

^b Percent difference compared with Day 1 ear thickness (average of both ears).

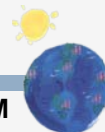
^c Average of both ears.

- **What dose should be selected for testing Chemical A and why?**
 - **5% should be tested because it is the highest dose that does not produce excessive local irritation (change in ear thickness <25% and erythema score < 3) and/or systemic toxicity (no clinical signs, body weight decrease <5%)**



Case Study 1: rLLNA Test

- You test Chemical A at 5% in AOO using the rLLNA
- In addition to Chemical A and the vehicle control, what other substance should be concurrently tested and how many animals should be used to test it?
 - The positive control, 25% hexyl cinnamic aldehyde (HCA), should be tested using 4 animals as recommended by the ICCVAM protocol and by OECD Test Guideline 429
- How many animals should be used in the vehicle control group?
 - 4 animals should be treated only with AOO, the vehicle control
- How many animals should be used in the Chemical A test group?
 - 4 animals should be treated with 5% Chemical A in AOO
- What is the reduction in the number of animals using the rLLNA compared with the three-dose LLNA?
 - 8 fewer animals (40% [8/20]). The rLLNA uses 12 animals. The three-dose LLNA uses 20 animals



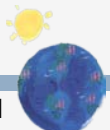
Case Study 1: rLLNA Data - 1

Group	Animal	DPM	Mean	SD	SEM	SI
Vehicle Control	1	175	300	143	71	
	2	225				
	3	300				
	4	500				
25% HCA ¹	5	1253	2531	946	473	
	6	2404				
	7	3080				
	8	3388				
5% Chemical A	9	350	488	155	77	
	10	400				
	11	500				
	12	700				

- Do any DPM values seem to be outliers?
- No statistical outliers were identified using Dixon's test

- Calculate the stimulation index (SI) values for each group
- $SI = \text{test substance mean dpm} / \text{vehicle control mean dpm}$

¹Hexyl cinnamic aldehyde



Case Study 1: rLLNA Data - 2

Group	Animal	DPM	Mean	SD	SEM	SI
Vehicle Control	1	175	300	143	71	1.00
	2	225				
	3	300				
	4	500				
25% HCA ¹	5	1253	2531	946	473	8.44
	6	2404				
	7	3080				
	8	3388				
5% Chemical A	9	350	488	155	77	1.62
	10	400				
	11	500				
	12	700				

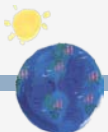
- Based on the positive control response, is the test acceptable?
 - Yes, the $SI \geq 3$

- Student's *t*-test indicated that there was no significant difference between the control group and the 5% Chemical A group ($p = 0.1253$)

¹Hexyl cinnamic aldehyde

Case Study 1: rLLNA Decision

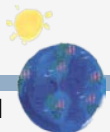
- Would you classify Chemical A (SI = 1.62) as a sensitizer or nonsensitizer and why?
 - Chemical A is a nonsensitizer because the SI < 3



Case Study 1: Summary and Breakout Group Discussion

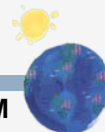
- This rLLNA case study demonstrated
 - The use of the rLLNA to test substances that are suspected to be nonsensitizers. Note that the rLLNA should also be used to test suspected sensitizers when dose-response information is not needed
 - Consideration and appropriate use of the rLLNA can decrease animal use by 40%
 - 80% of chemicals/products are **nonsensitizers** in standardized tests¹
 - The use of a prescreen test to determine the dose to be tested in the rLLNA
- Some labs get more consistent results using 35% HCA as the positive control
- How can you be sure that you are accurately identifying mild/moderate sensitizers if the positive control response dips near the 3.0 threshold?
- The variability of the positive control response should be monitored and evaluated over time
- Because EPA, Australia, etc., require 5 animals per group, tests for multiple regulatory entities should use most conservative protocol
- The SI is usually expressed to one decimal point, so the cutoff should be expressed as $SI < 3.0$
 - A result of 2.95 would round to 3.0; this is technically a nonsensitizer but other factors such as dose response and solubility could result in consideration as a weak sensitizer
- How should medical devices be tested?
 - What, other than rLLNA results, can be used to support a negative result?

¹Safford RJ. 2008. Reg Tox Pharmacol 51: 195-200.



Case Study 2: Introduction

- You have submitted a protocol to the IACUC to use the guinea pig maximization test to assess the ACD hazard potential of Chemical C
- The IACUC recommends the LLNA because it uses fewer animals and because positive responses do not produce pain and distress in the animals
- You respond to the IACUC that you cannot use the LLNA because your laboratory is not licensed to use radioactivity; you used this as a justification for the guinea pig test
- The IACUC notices that ICCVAM has recommended two nonradioactive LLNA methods, the LLNA: DA and the LLNA: BrdU-ELISA at <http://iccvam.niehs.nih.gov/> and asks you to consider these



Case Study 2: Prior Chemical Information

- You have the following information about Chemical C
 - Molecular weight <200
 - $\log K_{ow} = -0.66$
 - Soluble in water (up to 50%)
 - Only slightly soluble in AOO and *N,N*-dimethylformamide (DMF) and other organic solvents (up to 5%)
 - A structurally similar chemical, D, does not contain nickel and is not a potent ATP inhibitor or an ATP degrading enzyme
 - No other information is available
- Should you use the LLNA: DA or LLNA: BrdU-ELISA?
 - It depends on the substance to be tested and the equipment available. You decide to use the LLNA: DA because the substance does not contain nickel and it is not expected to be a potent ATP inhibitor or an ATP degrading enzyme. A luminometer is readily available.



Case Study 2: Prior Chemical Information

- Is there sufficient information to determine the maximum dose for testing in the LLNA: DA or should a prescreen test be performed?
 - The maximum dose tested must be the maximum concentration that does not produce overt systemic toxicity or excessive local skin irritation in the mouse
 - All existing toxicological information (i.e., acute toxicity and dermal irritation), structural information, and physicochemical information on Chemical C (and/or a structurally related substance) should be considered
 - A prescreen test must be performed because there is no information on the doses of Chemical C that produce systemic toxicity or local skin irritation



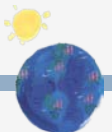
Case Study 2: Prescreen Test

- What vehicle should be selected for prescreen testing?
 - 1% Pluronic[®] L92, a surfactant, or the equivalent, because Chemical C is very hydrophilic. Pluronic[®] L92 will allow Chemical C to adhere to the dorsum of the ear
- What concentrations should be selected for prescreen testing?
 - 50%, which is the maximum soluble concentration in water, and then 25% and 10%, from the recommended dose series
- For the prescreen study, four topical applications of each dose of Chemical C were given to each of two mice using the LLNA: DA treatment schedule (days 1, 2, 3, and 7)
- One hour prior to each application, the mice were pre-treated with 1% sodium lauryl sulfate applied to the dorsum of the ear



Case Study 2: Prescreen Results

- No clinical signs of toxicity were observed at any dose
 - Body weight, ear thickness, and ear erythema data are shown on the next slide



Case Study 2: Prescreen Data

Chemical C Dose	Animal	Change in Body Weight ^a	Change in Ear Thickness Day 3 ^b	Change in Ear Thickness Day 7 ^b	Change in Ear Thickness Day 8 ^b	Erythema Score Day 1 ^c	Erythema Score Day 3 ^c	Erythema Score Day 7 ^c	Erythema Score Day 8 ^c
10%	1	+5.2%	9.8%	9.5%	9.5%	0	0	0	0
	2	+12.5%	10.7%	10.2%	10.3%	0	1	0	1
25%	3	+7.9%	20.2%	16.9%	17.5%	0	1	1	2
	4	+8.9%	16.9%	18.9%	19.4%	0	2	1	1
50%	5	+3.5%	25.2%	31.1%	32.1%	0	3	2	3
	6	+ 5.5%	34.1%	32.4%	33.7%	0	3	3	3

Erythema score: 0 = no erythema; 1 = very slight erythema (barely perceptible); 2 = well-defined erythema; 3 = moderate to severe erythema.

^a Percent difference of Day 7 body weight compared to Day 1 body weight.

^b Percent difference compared with Day 1 ear thickness (average of both ears).

^c Average of both ears.

- What doses should be selected for LLNA: DA testing?
 - 25% as the maximum because it was the highest dose that did not produce excessive local irritation or systemic toxicity
 - Change in ear thickness <25%, erythema score < 3, and no systemic toxicity (no clinical signs, body weight decrease <5%)
 - 10% and 5% should be the lower doses (recommended by protocol)



Case Study 2: LLNA: DA Data

Group	Animal	Relative Luminescence Units ¹	Mean	SD	SEM	SI
Vehicle Control	1	15218	27188	10027	5014	1.00
	2	22764				
	3	33905				
	4	36866				
5% Chemical C	5	24319	36534	10199	5099	1.34
	6	32753				
	7	41322				
	8	47742				
10% Chemical C	9	20851	31201	10875	5438	1.15
	10	27887				
	11	29565				
	12	46499				
25% Chemical C	13	20734	30030	10456	5228	1.10
	14	21245				
	15	38401				
	16	39741				

- Do any values seem to be outliers?
 - No statistical outliers were identified using Dixon's test
- Calculate the SI values for each group

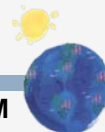
■ **SI = test substance mean RLU/vehicle control mean RLU**

¹Mean of two replicates



Case Study 2: LLNA: DA Decision

- For the positive control, 25% HCA, $SI = 3.92$
- Based on the positive control response, is the test acceptable?
 - Yes, the $SI > 1.8$, which is the criterion for potential skin sensitizers
- ANOVA of the the log-transformed relative luminescence units yielded $F = 0.5666$, $p = 0.6475$
- Based on a maximum SI of 1.34, would you classify Chemical C as a sensitizer or nonsensitizer and why?
 - Chemical C is a nonsensitizer because the maximum $SI < 1.8$. For the LLNA: DA, substances with $SI \geq 1.8$ are potential skin sensitizers



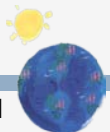
Case Study 2: Summary and Breakout Group Discussion

- This case study provided an example of
 - Use of the LLNA: DA
 - Dose selection for the LLNA: DA using prescreen data
 - A test where the LLNA: DA SI < 1.8
- Experience with one or the other assay (i.e., LLNA: BrdU-ELISA) would be a factor in choosing the assay, as would whether the lab had validated the assay
- Pretreatment with 1% SLS a unique feature of the LLNA: DA used to increase sensitivity of the assay
 - Pluronic L92 is a surfactant also, would it do the same thing? Do you need SLS pretreatment if Pluronic L92 is used as the vehicle? This is an important practical point for further study.
- No “equivalent” of Pluronic L92 is known at this time; has SLS been tested using Pluronic L92 as a vehicle?
- Does the 1% SLS pretreatment impact aqueous/nonaqueous vehicle performance?
- If you had an outlier and excluded it, you’d have only 3 animals in that group
 - Would that be an acceptable test? The relevant regulatory agency should be consulted.
- If a substance is completely soluble in an organic vehicle (e.g., 10%) but makes a suspension in an aqueous vehicle at a higher concentration (e.g., 25%), which is the best solution for testing?



Case Study 3: Introduction

- You have submitted a protocol to the IACUC to use the Buehler test to assess the ACD hazard potential of Chemical E
- The IACUC recommends the LLNA because it uses fewer animals and because positive responses do not produce pain and distress in the animals
- You cannot use the LLNA because your laboratory is not licensed to use radioactivity, but you notice that ICCVAM has recommended two nonradioactive LLNA methods at <http://iccvam.niehs.nih.gov/>



Case Study 3: Prior Chemical Information

- You have only the following information on Chemical E
 - Molecular weight <170
 - $\log K_{ow} = 2.86$
 - A structurally similar substance, Chemical F, is a sensitizer
 - The maximum concentration for testing Chemical E should be 25% in DMSO because it was the maximum soluble concentration that did not produce systemic toxicity or excessive local irritation
- Should you use the LLNA: DA or LLNA BrdU-ELISA?
 - It depends on the substance to be tested and the equipment available. You decide to use the LLNA: BrdU-ELISA because you are familiar with ELISA techniques and you have access to a microplate reader



Case Study 3: LLNA: BrdU-ELISA Data

Group	Animal	Absorbance ¹	Mean	SD	SEM	SI
Vehicle Control	1	0.119	0.243	0.221	0.110	1.00
	2	0.123				
	3	0.157				
	4	0.573				
5% Chemical E	5	0.171	0.227	0.048	0.024	0.93
	6	0.208				
	7	0.279				
	8	0.251				
10% Chemical E	9	0.089	0.200	0.102	0.051	0.82
	10	0.157				
	11	0.226				
	12	0.327				
25% Chemical E	13	0.197	0.296	0.122	0.061	1.22
	14	0.245				
	15	0.269				
	16	0.474				

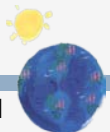
- Calculate the SI values for each group

- $SI = \frac{\text{test substance mean abs}}{\text{vehicle control mean abs}}$

¹Mean of three replicates

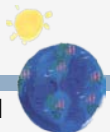
Case Study 3: Evaluation of LLNA: BrdU-ELISA Data

- For the positive control, 25% HCA, $SI = 2.44$
- Based on the positive control response, is the test acceptable?
 - Yes, the $SI > 1.6$, which is the criterion for potential skin sensitizers
- SI values were 0.93 at 5%, 0.82 at 10%, and 1.22 at 25%. Would you classify Chemical E as a sensitizer or nonsensitizer and why?
 - For the LLNA: BrdU-ELISA, substances with $SI \geq 1.6$ are potential skin sensitizers. Based on these data, Chemical E appears to be a nonsensitizer because the maximum $SI < 1.6$, however. . .



Case Study 3: Evaluation of Extreme Values

- After looking at the data your study director was surprised that Chemical E was negative because similar products, including Chemical F, were sensitizers
- The study director suggested that an outlier test be performed
 - Dixon's test indicated that the extreme value in the vehicle control group, 0.573, was an outlier at $p < 0.01$ among the 4 values in the vehicle control group
 - 0.573 was also an outlier at $p < 0.001$ among the 24 values in the historical vehicle control database for DMSO
 - The other two extreme values were not outliers
- You exclude the outlier and recalculate the SI values



Case Study 3: LLNA: BrdU-ELISA Data Without Outlier

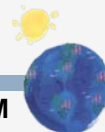
Group	Animal	Absorbance ¹	Mean without Outlier	SD	SEM	SI without Outlier
Vehicle Control	1	0.119	0.133	0.021	0.012	1.00
	2	0.123				
	3	0.157				
	4	0.573				
5% Chemical E	5	0.171	0.227	0.048	0.024	1.71
	6	0.208				
	7	0.279				
	8	0.251				
10% Chemical E	9	0.089	0.200	0.102	0.051	1.50
	10	0.157				
	11	0.226				
	12	0.327				
25% Chemical E	13	0.197	0.296	0.122	0.061	2.22
	14	0.245				
	15	0.269				
	16	0.474				

- Calculate the SI values for each group
- $SI = \frac{\text{test substance mean abs}}{\text{vehicle control mean abs}}$

¹Mean of three replicates

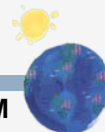
Case Study 3: LLNA: BrdU-ELISA Decision

- SI values with the outlier were 0.93 at 5%, 0.82 at 10%, and 1.22 at 25%
- SI values when excluding the outlier were 1.71 at 5%, 1.50 at 10%, and 2.22 at 25%
- Would you classify Chemical E as a sensitizer or nonsensitizer and why?
 - Chemical E is a sensitizer because the maximum SI = 2.22, which is >1.6 . For the LLNA: BrdU-ELISA, substances with $SI \geq 1.6$ are potential skin sensitizers



Case Study 3: Summary and Breakout Group Discussion

- This example shows that an outlier in the vehicle control group can produce erroneous results that may impact the classification of a substance using the LLNA: BrdU-ELISA
- In reviewing LLNA tests, NICEATM has also observed extreme low values in test substance groups that may produce false negative results
 - This emphasizes the need to collect individual animal data in order to identify outliers that could yield false negative results
- Structural similarities are of limited use when predicting sensitization potential
 - May be more useful when considering acute toxicity (i.e., when evaluating whether a prescreen is necessary)
- The absorbances in the historical vehicle control database should be evaluated
- A clear dose response is lacking, so the results are still questionable
- Other extreme values in the treatment groups may not be outliers, but may impact the evaluation as well (e.g., one high value in the high dose group)
- The number of cells or total protein applied to the wells isn't standardized and could be a source of variation
 - Consistency in processing the lymph nodes is very important



Case Study 4: Introduction and Prior Chemical Information

- You wish to assess the ACD hazard potential of another substance, Chemical G, using the LLNA: DA
- You have only the following information about Chemical G
 - Molecular weight = 100-150
 - $\log K_{ow} = 2.86$
 - More soluble in AOO than DMF or other recommended organic solvents
 - Peptide reactivity is minimal
 - h-CLAT result is positive
- You use the LLNA: DA at a maximum concentration of 50%, the highest soluble concentration that did not produce excessive local irritation or systemic toxicity

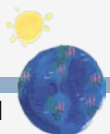


Case Study 4: LLNA: DA Data

Group	Animal	Relative Luminescence Units ¹	Mean	SD	SEM	SI
Vehicle Control	1	15187	20576	5546	2773	1.00
	2	18744				
	3	20074				
	4	28298				
10% Chemical G	5	19026	25167	4299	2149	1.22
	6	25653				
	7	27127				
	8	28861				
25% Chemical G	9	27846	40921	10986	5448	1.99
	10	36281				
	11	47941				
	12	51618				
50% Chemical G	13	38134	49037	8244	4122	2.38
	14	47782				
	15	52938				
	16	57296				

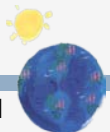
- Do any values seem to be outliers?
 - No statistical outliers were identified using Dixon's test
- Calculate the SI values for each group
- $SI = \frac{\text{test substance mean RLU}}{\text{vehicle control mean RLU}}$

¹Mean of two replicates



Case Study 4: Supporting Information and LLNA: DA Decision

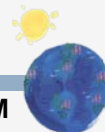
- ANOVA of the log-transformed relative luminescence units: $F = 12.61$, $p = 0.0005$
- Dunnett's test
 - 10% - $q = 1.322$, $p > 0.05$
 - 25% - $q = 4.210$, $p < 0.05$
 - 50% - $q = 5.4297$, $p < 0.05$
- Additional data
 - Minimal peptide reactivity
 - Positive h-CLAT
- The maximum SI = 2.38. Would you classify Chemical G as a sensitizer or nonsensitizer and why?
 - Chemical G is a sensitizer because the maximum SI > 1.8



Case Study 4: LLNA: DA Interpretation

- 25% (3/12) of the nonsensitizers in the validation database were false positive with $1.8 < SI < 2.5$. Do you have information that suggests the Chemical G results might be false positive?
 - Only the minimal peptide reactivity could possibly be used to suggest that the LLNA: DA result is false positive. 12% (6/52) of the sensitizers evaluated had minimal peptide reactivity¹
 - The LLNA: DA result and h-CLAT result support a true positive result
 - Although $SI = 2.38$ is in the range where false positives may occur, the preponderance of the evidence supports the sensitizer classification

¹Gerberick et al. 2007. Toxicol Sci 97: 417-427.



Case Study 4: Summary

- The purpose of this LLNA: DA example was to demonstrate how to interpret LLNA: DA results when the SI value is between 1.8 and 2.5, the range where false positive results may occur

