January 20, 2011

ICCVAM Workshop Series on Best Practices for Regulatory Safety Testing

Assessing the Potential for Chemically Induced Allergic Contact Dermatitis

New Models in the Validation Pipeline for ACD Hazard Testing
human Cell Line Activation Test: h-CLAT

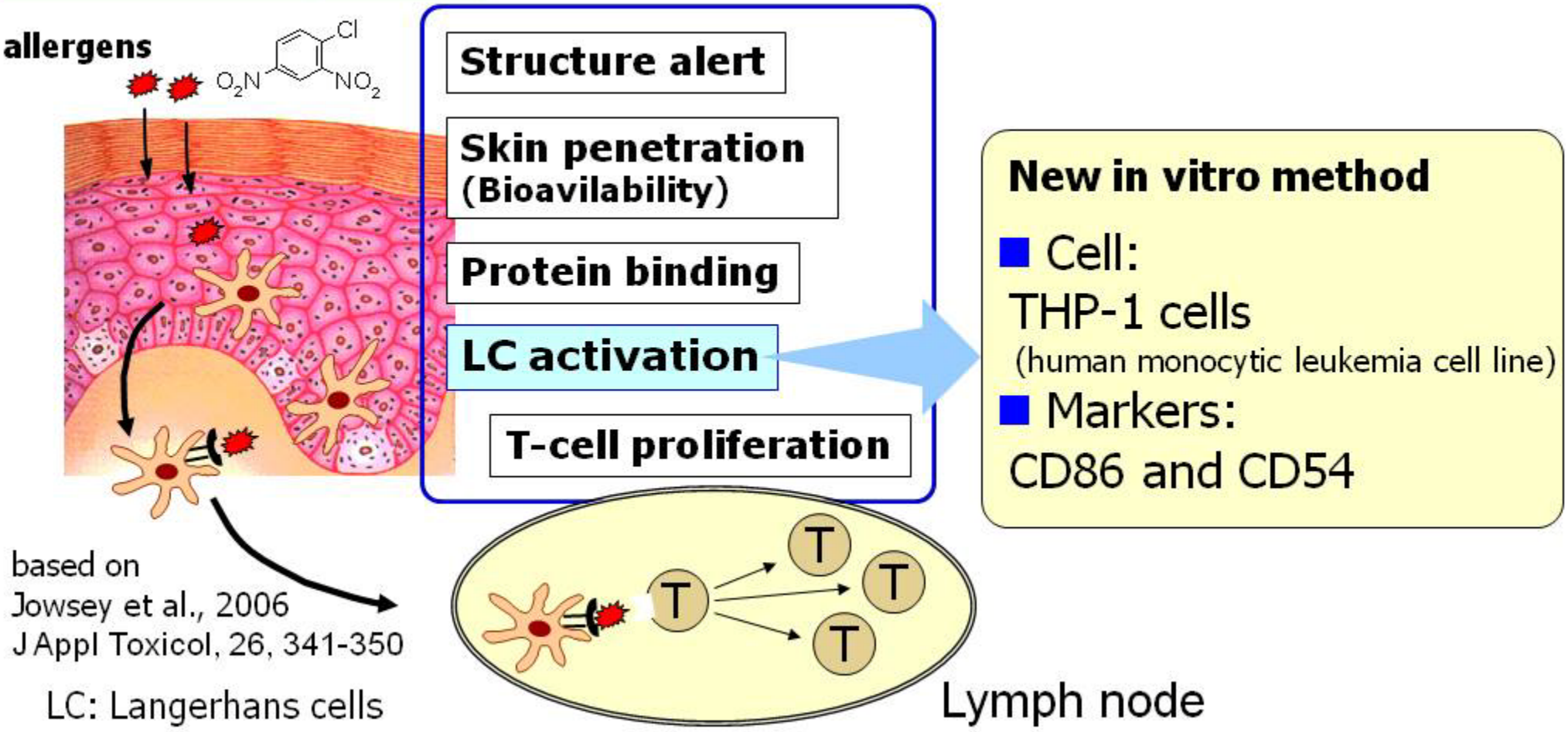
Hitoshi Sakaguchi, Kao Corporation

Takao Ashikaga, Shiseido Co., Ltd

Approach for Developing of in vitro Methods

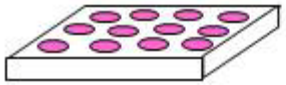
It is imperative to understand the mechanisms the sensitization (induction) phase of contact hypersensitivity (Vandebriel et al., 2005)

Induction phase



Human Cell Line Activation Test (h-CLAT)*

Procedure



THP-1
1x10⁶ cells /mL

24h

Culture with chemicals,
8 doses based on CV75

FcR blocking

Cell staining (CD86 & CD54)

Flow cytometric analysis

Relative Fluorescence Intensity (RFI)

$$\text{RFI} = \frac{\text{MFI of chemical treated cells} - \text{MFI of chemical treated Isotype control cells}}{\text{MFI of vehicle control cells} - \text{MFI of vehicle Isotype control cells}} \times 100$$

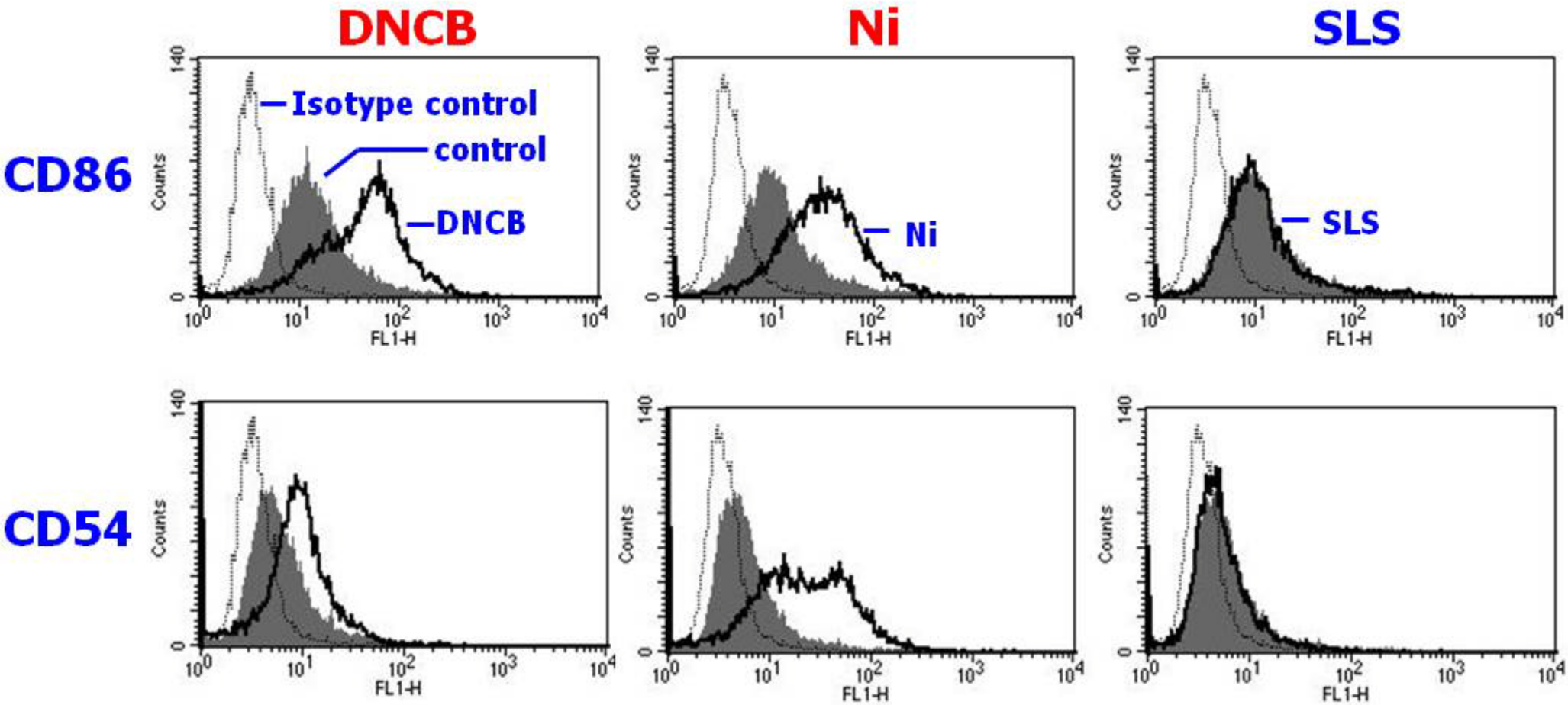
MFI = geometric mean fluorescence intensity

Prediction Model

- Viability ≥ 50% by Propidium Iodide
- Positive criteria: CD86 RFI ≥ 150% and/or CD54 RFI ≥ 200%
- Positive: 2 of 3 independent data at any dose should exceed the positive criteria

*: Ashikaga et al., 2006 Toxicol In Vitro 767-73., Sakaguchi et al., 2006 Toxicol In Vitro 774-84.

Histogram of CD86 / CD54 expression



DNCB and Ni (typical allergens) enhanced both CD86 and CD54 expressions but SLS (non-allergen) did not.

Miyazawa et al., Toxicology in Vitro 2007

Today's presentation

- **Predictive capacity**
  - Evaluation of 117 chemicals by the h-CLAT to compare with LLNA
- **Applicability domain**
  - Applicability domain based on the data base
- **Classification of skin sensitization potency**
  - Using EC150 and EC200 values as the indicator
- **Inter-laboratory study**
  - Ring Trials in the COLIPA (5 labs) and Japan (7 labs)

Results of 117 test chemicals

Chemical	Ames		Human Risk	h-CLAT					
	Potency category	EC(IC)[%]		Result	CD86	CD54	EC150 (Y: mM)	EC300 (Y: mM)	CV75 (μg/ml)	vehicle
Diphenylcyclopropenone	Extreme	0.003	P	P	-	+	-	3.02	6.0	DMSO
Oxazolone		0.003	P	P	+	-	2.71	-	189.8	DMSO
MCI/MI (act.: 1.5%)		0.005	P	P	+	-	2.21	-	3.2	Saline
Bandrowski's base		0.008		P	+	+	3.65	2.8	2.3	DMSO
p-Benzoquinone		0.0099	P	P	+	+	2.68	2.25	4.3	DMSO
1-Benzoylacetone		0.04	P	P	-	+	-	36.6	62.8	DMSO
Tetrachlorosalicylanilide		0.04		P	-	+	-	2.68	6.3	DMSO
2,4-Dinitrochlorobenzene: DNCB		0.05	P	P	+	+	2.3	2.68	5.0	DMSO
4-Nitrobenzyl bromide		0.05		P	+	+	0.65	0.61	3.6	DMSO
Potassium dichromate		0.08	P	P	+	+	2.09	1.05	3.2	Saline
Glutaraldehyde (act.: 50%)	Strong	0.1	P	P	+	+	2.78	2.7	5.3	Saline
1,4-Dihydroquinone		0.11	P	P	+	-	2.13	-	5.0	Saline
1,4-Phenylenediamine		0.18	P	P	+	-	2.09	-	39.7	Saline
Maleic anhydride		0.18	P	P	-	+	-	258.8	858.0	DMSO
Phthalic anhydride		0.18	P	N	-	-	-	-	>4000	DMSO
Benzyl bromide		0.2	P	P	+	+	3.2	2.85	7.5	DMSO
Benzoyl peroxide		0.30	P	N	-	-	-	-	40.0	DMSO
Geeryl gallate		0.30	P	P	+	+	2.02	2.01	8.2	DMSO
Propyl gallate		0.32	P	P	-	+	-	32.5	125.0	DMSO
Cobalt chloride		0.38	P	P	-	+	-	35.5	208.3	Saline
2-aminophenol		0.4	P	P	+	-	0.80	-	8.0	DMSO
Chloramine T		0.4	P	P	+	+	2502	201	304.7	Saline
2-Nitro-1,4-phenylenediamine		0.5	P	P	-	+	-	278.7	480.7	DMSO
CDI		0.6		P	+	+	10.73	18.37	28.5	Saline
Formaldehyde (act.: 37%)		0.61	P	P	+	+	4.3	5.16	5.8	Saline
Iodopropynyl butylcarbamate		0.87		P	-	+	-	8.15	12.8	DMSO
Methyldibromo glutaronitrile	Moderate	0.9	P	P	+	+	1.08	0.62	0.9	DMSO
Isoeugenol		1.2	P	N	-	-	-	-	112.5	DMSO
1-Naphthol		1.3		P	+	+	1.83	12.7	57.2	DMSO
1-Phenyl-1,2-propanedione		1.3		P	+	+	80.5	59.7	123.1	DMSO
Glyoxal (act.: 40%)		1.4	P	P	-	+	-	288.7	368.0	Saline
2-Hydroxyethyl acrylate		1.4	P	P	+	+	1.08	1.88	2.8	Saline
Vinyl pyridine		1.6		P	+	-	13.2	-	72.8	DMSO
2-Mercaptobenzothiazole		1.7	P	P	-	+	-	57.5	168.0	DMSO
Methylisothiazolinone (act.:1.7%)		1.8	P	P	+	+	0.73	7.88	28.7	Saline
3-Dimethylaminopropylamine		2.2	P	P	+	+	1.31	18.58	278.7	Saline
Ethylene diamine		2.2	P	P	+	-	28.57	-	271.7	Saline
1,2-Benzisothiazolin-3-one		2.3	P	P	-	+	-	0.58	1.83	DMSO
Methyl-2-nonynoate		2.5	P	P	+	-	60.8	-	180.7	DMSO
Cinnamic aldehyde		3.0	P	P	+	+	10.2	12.3	28.0	DMSO
Phenylacetaldehyde		3.0	P	P	+	+	11.5	13.6	27.0	DMSO
3-Aminophenol		3.2	P	P	-	+	-	11.71	268.2	DMSO
Diethyl sulfate		3.3		P	+	-	28.06	-	373.0	DMSO
Benzylidene acetone		3.7		P	+	+	25.8	34.6	35.3	DMSO
3-Propylidenephthalide		3.7		P	+	+	30.2	55.6	113.0	DMSO
2,4-Hexadienal		4.0		P	+	+	3.51	5.09	8.9	DMSO
a-Methyl cinnamic aldehyde		4.5		P	+	+	40.3	70.16	138.0	DMSO
Nickel sulfate		4.8	P	P	+	+	62.2	65.3	150.0	Saline
Tetramethylthiuramdisulfide		5.2	P	P	+	+	0.11	2.68	10.0	DMSO
trans-2-Hexenal		5.5		P	+	+	8	5.7	18.8	DMSO
3,4-Dihydrocoumarin		5.8		P	-	+	-	538.7	810.0	DMSO
Resorcinol		5.7	N	P	-	+	-	200.0	813.4	Saline
Diethylenetriamine		5.8	P	N	-	-	-	-	1221.8	Saline
Diethyl maleate		5.8	P	P	+	-	88	-	128.0	DMSO
2-Methoxy-4-methyl-phenol		5.8		P	+	+	30.8	207.6	280.0	DMSO
2-Phenylpropionaldehyde		6.3		P	+	+	38.2	66.5	97.0	DMSO
4-Chloroaniline		6.5	P	P	+	-	87.7	-	200.0	DMSO
Perillaldehyde		8.1		P	+	-	51.43	-	68.3	DMSO

Chemical	Ames		Human Risk	h-CLAT					
	Potency category	EC(IC)[%]		Result	CD86	CD54	EC150 (Y: mM)	EC300 (Y: mM)	CV75 (μg/ml)	vehicle
1-Bromohexane	Weak	10		N	-	-	-	-	1,818.3	DMSO
Amyl cinnamic aldehyde		11	P	P	-	+	-	25.88	28.2	DMSO
Hexyl cinnamic aldehyde		11	P	N	-	-	-	-	37.0	DMSO
2,3-Butanedione		11		P	+	+	2.68	6.8	9.8	Saline
Citral		13	P	P	+	+	8.81	15	28.0	DMSO
Eugenol		13	P	P	+	+	8.68	137.2	1,401.2	DMSO
Abietic acid		15	P	N	-	-	-	-	88.8	DMSO
Oxalic acid		15		P	+	-	30.0.30	-	>1000	DMSO
Benzyl benzoate		17		N	-	-	-	-	287.2	DMSO
4-Allylanisole		18		P	-	+	-	207.8	1,881.0	DMSO
Citral		19	P	P	-	+	-	6.6.5	82.2	DMSO
Phenyl benzoate		20	P	P	+	+	188.2	238.8	>5000	DMSO
Cinnamic alcohol		21	P	P	+	+	1.67	188.8	3,501.0	DMSO
Benzocaine		22	P	P	+	-	7.8.2	-	5,65.5	DMSO
Cyclamen aldehyde		22	P	N	-	-	-	-	68.8	DMSO
Imidazolidinyl urea		28	P	P	+	+	8.2.3	38.3	80.1	Saline
Geraniol		28	P	P	+	-	128.3	-	1,681.0	DMSO
5-Methyl-2,3-hexanedione		28		P	+	+	25.3.88	2.1.1.88	2,58.1	DMSO
Ethyleneglycol dimethacrylate		28	P	P	-	+	-	5.35.68	5,03.8	DMSO
Linalool		30	P	P	-	+	-	8.8.3	2,80.0	DMSO
Penicillin G		30	P	P	-	+	-	3.7.5.6.3	>5000	Saline
Butyl glycidyl ether		31	P	N	-	-	-	-	1,85.3	DMSO
Hydroxycitronellal		33	P	P	+	+	3.5.8	2.8.8	7,00.0	DMSO
Pyridine		72	P	P	+	-	2370	-	4,08.8.7	Saline
Aniline		80	P	P	+	+	9.2.7.8	5.5.0.8	8,30.0	DMSO
Nonanoic acid		21(FP)		P	+	-	1.3.8.68	-	2,68.8	DMSO
Avermectin	Non-sensitizer	NC	N	N	-	-	Not done		8,28.0	DMSO
2-Acetylcyclohexanone				P	-	+			8,23.0	DMSO
Benzaldehyde				N	-	-			8,88.8	DMSO
Benzalkonium chloride				N	-	-			3.0	Saline
Benzoic acid				N	-	-			>1000	DMSO
1-Bromobutane			N	P	+	+			5,00.0	DMSO
1-Butanol				N	-	-			>1000	DMSO
Chlorobenzene			N	P	+	-			5,07.2	DMSO
Coumarin				N	-	-			5,37.8	DMSO
Dextran			N	N	-	-			>5000	Saline
Diethyl phthalate			N	P	-	+			8,00.0	DMSO
Dimethyl formamide				N	-	-			>5000	Saline
Ethyl benzylacetate				N	-	-			5,71.1	DMSO
Ethyl vanillin			N	N	-	-			5,88.5	DMSO
Glycerol			N	N	-	-			>5000	Saline
4-Hydroxybenzoic acid			N	N	-	-			>1000	DMSO
2-Hydroxypropyl methacrylate			N	N	-	-			>1000	DMSO
Isopropanol			N	N	-	-			>5000	Saline
Lactic acid			N	N	-	-			2800	Saline
6-Methyl coumarin			N	N	-	-			2,78.8	DMSO
Methyl salicylate			N	N	-	-			5,62.4	DMSO
Octanoic acid			N	P	-	+			352	DMSO
Propylene glycol			P	N	-	-			>1000	Saline
Propyl paraben			P	P	+	+			1,08.7	DMSO
Saccharin				N	-	-			>1000	DMSO
Salicylic acid			N	P	-	+			>1000	DMSO
Streptomycin sulfate				N	-	-			>1000	Saline
Sulfanilamide				N	-	-			>1000	DMSO
Tween 80			N	N	-	-			>5000	Saline
Vanillin			P	N	-	-			8,50.0	DMSO
Zinc sulphate			N	P	-	+			8,70.8	Saline
Sodium taurolsulfate	FP		N	N	-	-			80.0	Saline

Nukada et al., WC7 2009, Ashikaga et al., ATLA 2010, Nukada et al., ESCD 2010

Comparative evaluation with LLNA and human

h-CLAT vs LLNA

		h-CLAT	
		+(83)	-(34)
LLNA	+(85)	75	10
	-(32)	8	24

Sensitivity: 75/85 (88%)
Specificity: 24/32 (75%)
Positive predictivity: 75/83 (90%)
Negative predictivity: 24/34 (71%)
Accuracy: 99/117 (85%)

h-CLAT vs human

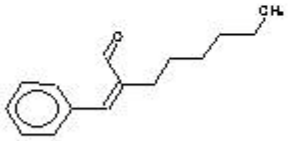
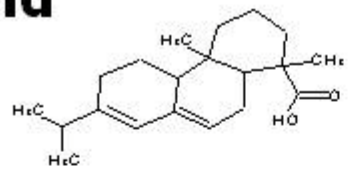
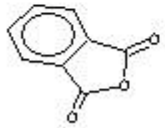
		h-CLAT	
		+(51)	-(20)
Human	+(55)	46	9
	-(16)	5	11

Sensitivity: 46/55 (84%)
Specificity: 11/16 (69%)
Positive predictivity: 44/51 (90%)
Negative predictivity: 11/20 (55%)
Accuracy: 57/71 (80%)

Good predictive capacity, but some false negative / positive

Nukada et al., WC7 2009, Ashikaga et al., ATLA 2010, Nukada et al., ESCD 2010

False negative (1) : Solubility

	Applying dose based on cytotoxicity		Estimated maximum water solubility*
Hexyl cinnamic aldehyde	44-12 μg/mL	>	2.75 μg/mL
	↳ Precipitation or cloudy solution		
Abietic acid	154-43 μg/mL	>	0.09 μg/mL
	↳ Precipitation or cloudy solution		
Phthalic anhydride	Max applying dose (400 μg/mL) with no-cytotoxicity		
	↳ To be dissolved in appropriate sol.		

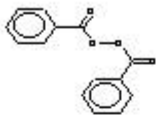
The chemical with poor water solubility is one of limitation

Ashikaga et al., ATLA 2010

*: Calculated with “Water frag” software.

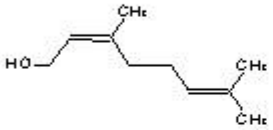
False negative (2): Pro(Pre)-hapten

Benzoyl peroxide



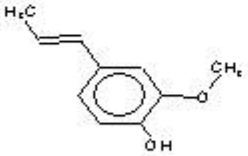
Metabolic activity can change the structure
(Nacht et al., J. Am. Acad. Dermatol., 4, 31-37, 1981).

Geraniol



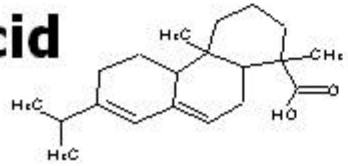
- Metabolic activity or air oxidation can change the structure (Basketter et al., Contact Dermatitis, 47(3), 161-164, 2002).
- Oxidation products of geraniol (Geranial and Neral) augmented CD54 expression (Kosaka et al., SOT 2008).

Isoeugenol



Oxidation involes senstitising potencial
(Bertrand et al., Chem Res Toxicol., 10(3), 335-343, 1997).

Abietic acid



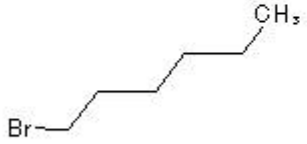
Air oxidation involves expression of sensitizing potencial
(Basketter et al., Food Chem Toxicol 33, 1051-1056, 1995).

The h-CLAT had limitation for some pro- and pre-hapten

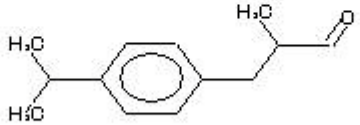
False negative (3) : Sensitivity

Weak sensitizers by LLNA classification

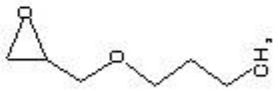
1-Bromohexane



Cyclamen aldehyde



Butyl glycidyl ether



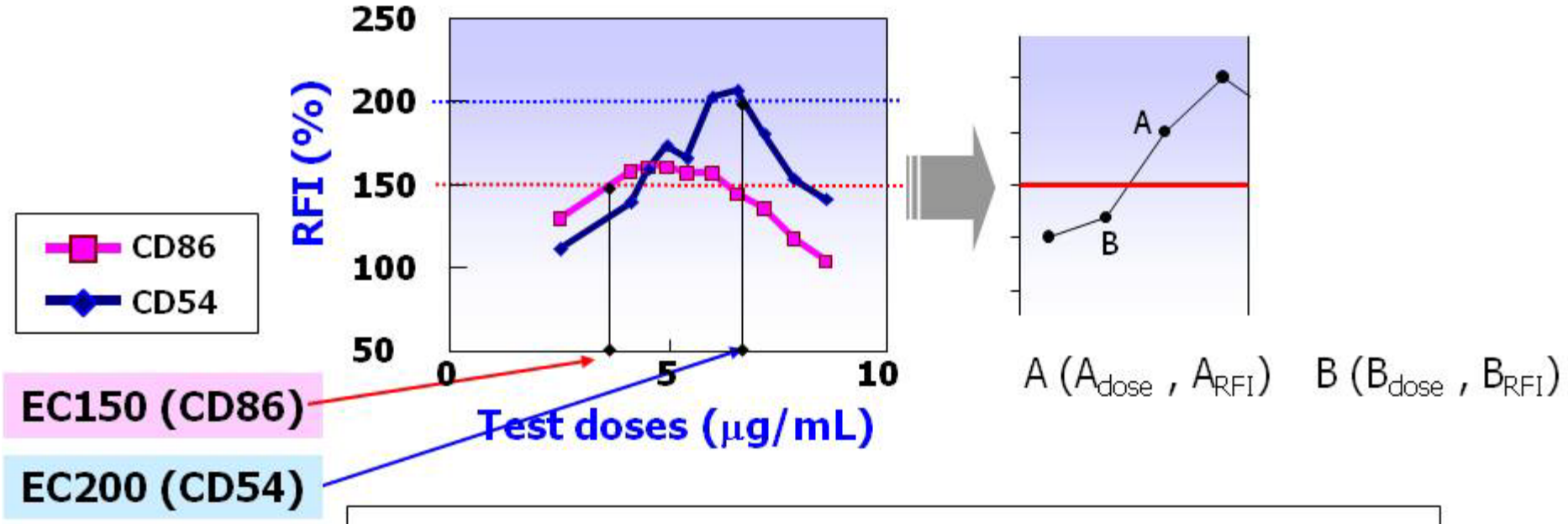
LLNA class	Number of tested chemicals	Number of false negatives	Sensitivity (%)
Extreme	8	0	100
Strong	16	2	88
Moderate	24	2	92
Weak	23	5	78

Several weak sensitizers could not enhance CD86/CD54 expression

Ashikaga et al., ATLA 2010

Correlation between h-CLAT and in vivo data

EC150 / 200 (Estimated concentration of RFI 150 / 200)



$$EC150\,(CD86) = B_{dose}\,[(150 - B_{RFI}) / (A_{RFI} - B_{RFI})] \times (A_{dose} - B_{dose})$$

$$EC200\,(CD54) = B_{dose}\,[(200 - B_{RFI}) / (A_{RFI} - B_{RFI})] \times (A_{dose} - B_{dose})$$

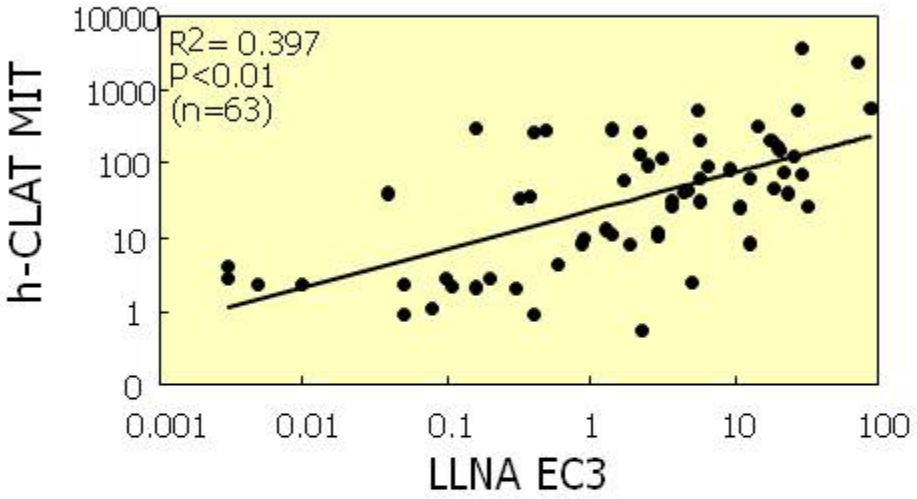
The intermediate value of three experiments was defined as EC150 or EC200.

Calculated based on the calculational procedure of LLNA EC3

Classification of skin sensitization potency

Minimum Induction Threshold of h-CLAT – MIT (h-CLAT) -

determined as a smaller value of either EC150 or EC200



Significant correlation with LLNA EC3

Might be useful to classify...

LLNA EC3 ...?
Proposed GHS subcategories ...?

Ref. Proposed GHS subcategories for skin sensitization based on LLNA EC3 and the example of prediction

Subcategory	Animal test results (using LLNA data)	Cut off (h-CLAT)	Accuracy(%)
1A (Strong sensitizer)	EC3 ≤ 2%	MIT 10 μg/mL	78.8
1B (Weak sensitizer)	EC3 > 2%		

COLIPA and Japanese Ring Trials

- **Purpose**
  - Protocol transferability
  - Inter-laboratory reproducibility
  - Predictive capacity
- **Goals**
  - Identify unexpected problems with either test design or procedures
    - Protocol optimization/standardization
  - Identify problems with data analysis / interpretation
    - Prediction model refinement
- **Members**
  - COLIPA: P&G, L'Orel, Henkel-Phnion, Shiseido and Kao
  - Japan: Kanebo Cosmetics, Kose, Lion, Nippon Menard Cosmetic, Pola Chemical Industries, Shiseido and Kao

COLIPA 4th Ring Trial summary data

Chemical	Potency	Lab B	Lab C	Lab D	Lab E
PPD	Strong	+ (2/3)	+ (3/3)	+ (3/3)	+ (3/3)
Methyldibromo glutaronitrile	Strong	+ (3/3)	+ (3/3)	+ (2/3)	+ (3/3)
2-Mercaptobenzothiazole	Strong	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
Cinnamic Aldehyde	Moderate	- (1/3)	+ (3/3)	+ (2/3)	+ (3/3)
Tetramethylthiuram Disulfide	Moderate	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
Glycerol	NS	- (0/3)	- (0/3)	- (0/3)	- (0/3)
Salicylic Acid	NS	+ (3/3)	+ (3/3)	+ (2/3)	+ (3/3)

7 test chemicals (5 allergens, 2 non-allergens), 4 labs

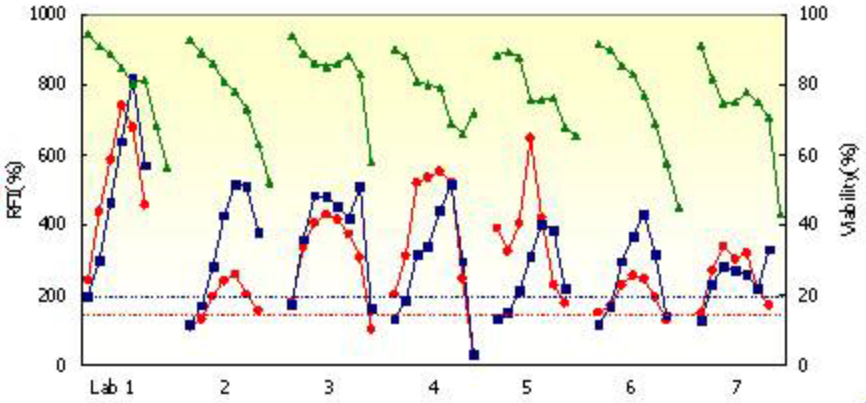
- Cinnamic Aldehyde : one false negative data
- Salicylic acid : false positive in all labs
- Good inter-laboratory reproducibility
- Almost good predict performance

Sakaguchi et al., Toxicology in Vitro 2010

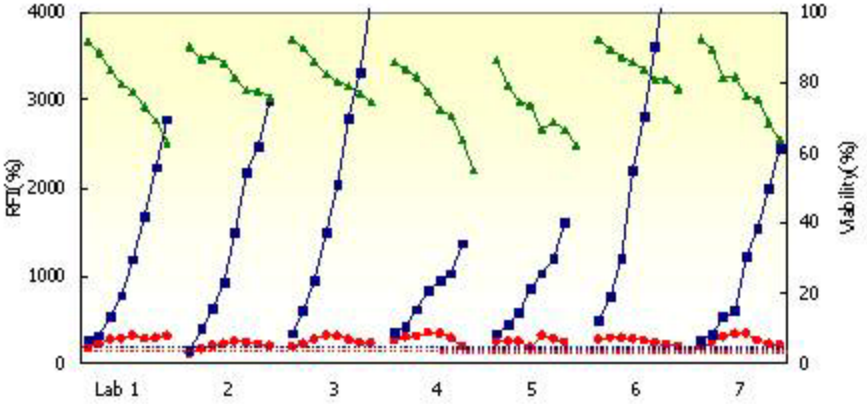
Japanese 1st Ring Trial

- 3 test chemicals (2 allergens, 1 non-allergen), 7 labs
- Test doses were same in all labs

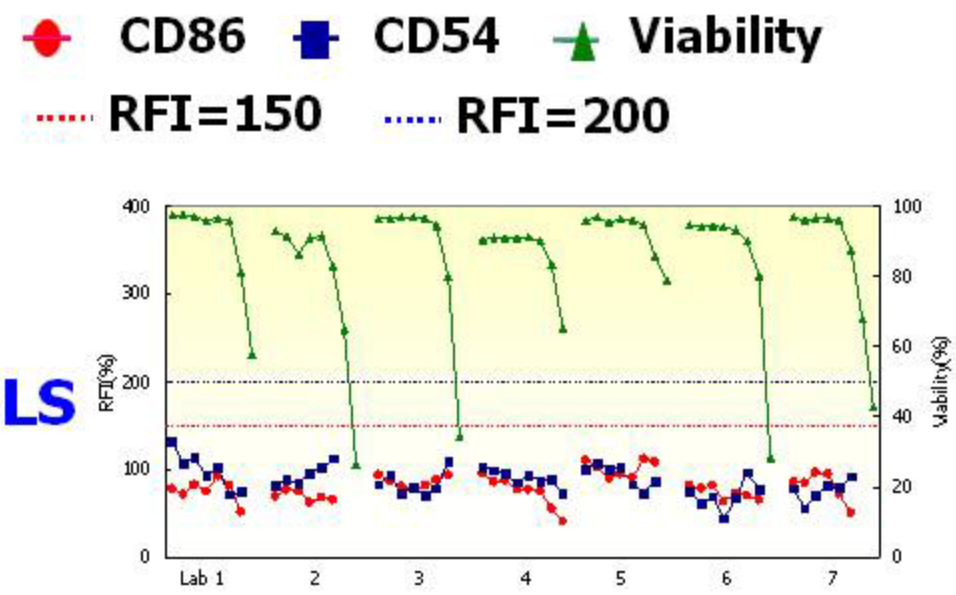
DNCB



Ni



SLS



- Good inter-lab reproducibility
- Good predict performance

Ashikaga et al., AATEX 2008

Summary

- **Predictive capacity (117 chemicals)**
  - Good prediction performance (accuracy: 85%/80% between the h-CLAT/human and LLNA) was observed.
- **Applicability domain**
  - Possible applicability domain was solubility, metabolic activity, sensitivity, etc.
- **Classification of skin sensitization potency**
  - MIT might be useful to predict the allergic potency of chemicals classified by GHS classification
- **Inter-laboratory study**
  - COLIPA : 15 chemicals, approx 85% predicted correctly
  - Japan : 8 chemicals, approx 96% predicted correctly
  - Good inter-lab reproducibility and predictive performance

ECVAM prevalidation study

- **Liaison:**
  - JaCVAM and ICCVAM
- **Test methods:**
  - Direct Peptide Reactivity Assay (DPRA)
  - Myeloid U937 Skin Sensitization Test (MUSST)
  - **human Cell Line Activation Test (h-CLAT)**
- **Main purpose**
  - The assessment of the robustness and reliability
- **Experimental design**
- 24 coded chemicals in three (or four) laboratories each for the assessment of the within- and between-laboratory reproducibility

Acknowledgments

Shiseido Co., Ltd

Sakiko Sono

Makie Ishikawa

Katsurako Yoneyama

Morihiko Hirota

Shigenobu Hagino

Hiroshi Itagaki

Kao Corporation

Masaaki Miyazawa

Yuuko Nukada

Nanae Kosaka

Yukiko Terada

Yuuichi Ito

Hiroyuki Suzuki

Thank you for your attention