Table 2: Reference test chemicals used in the validation study

No.	Chemical	Supplier	CAS number	GHS label	*In vivo* score
a) In the first phase					
I	Ethanol	WPCI	64-17-5	No cat.	0
II	Glycerol	Sigma-Aldrich	56-81-5	No cat.	0
III	Naphthalene acetic acid	WPCI	86-87-0	No cat.	0
IV	Sodium lauryl sulphate (SLS) 5% v/v (positive control)	WPCI	151-21-3	Not applicable	
b) In the second phase					
1	1-Bromo-4-chlorobutane	WPCI	6940-78-9	No cat.	0.0
2	Diethyl phthalate	WPCI	84-66-2	No cat.	0.0
3	di-Propylene glycol	WPCI	25265-71-8	No cat.	0.0
4	Naphthalene acetic acid	WPCI	86-87-3	No cat.	0.0
5	Allyl phenoxyacetate	WPCI	7493-74-5	No cat.	0.3
6	Isopropanol	WPCI	67-63-0	No cat.	0.3
7	4-Methylthio-benzaldehyde	WPCI	3446-89-7	No cat.	1.0
8	Methyl stearate	KCC	112-61-8	No cat.	1.0
9	Allyl heptanoate	WPCI	142-19-8	No cat.	1.7
10	Heptyl butyrate	Sigma-Aldrich	5870-93-9	No cat.	1.7
11	Hexyl salicylate	Sigma-Fluka	6259-76-3	No cat.	2.0
12	Terpinyl acetate	Alfa Aesar	80-26-2	No cat.	2.0
13	*Sodium lauryl sulphate (SLS) 5% w/v	WPCI	151-21-3	Not applicable	
14	1-Decanol	WPCI	112-30-1	Category 2	2.3
15	Cyclamen aldehyde	WPCI	103-95-7	Category 2	2.3
16	1-Bromohexane	WPCI	111-25-1	Category 2	2.7
17	α-Terpineol	KCC	98-55-5	Category 2	2.7
18	di-*n*-Propyl disulphide	WPCI	629-19-6	Category 2	3.0
19	Butyl methacrylate	WPCI	97-88-1	Category 2	3.0
20	Heptanal	KCC	111-71-7	Category 2	4.0
c) In the third phase					
21	Cinnamaldehyde	Sigma-Aldrich	104-55-2	No cat.	2.0
22	2-Chroromethyl-3,5-dimethyl-4-methoxypyridine HCl	WPCI	322-76821	Category 2	2.7
23	Potassium hydroxide (5% w/v)	WPCI	168-21815	Category 2	3.0
24	Benzenethiol 5-(1,1-dimethyethyl)-2-methyl	TCI	7340-90-1	Category 2	3.3
25	1-Methyl-3-phenyl-1-piperazine	TCI	5271-27-2	Category 2	3.3
26	1,1,1-Trichloroethane	WPCI	200-02463	Category 2	4.0

Replacement for tri-isobutyl phosphate, and also used as the positive control.

KCC = Kanto Chemical Co. Inc.; TCI = Tokyo Chemical Industry Co. Ltd; WPCI = Wako Pure Chemical Industries Ltd; No cat. = no category.

of the tissue when exposed to the negative control, and viability of the tissue were assessed, via the MTT assay, after exposure to various concentrations of an SLS solution for 18 hours.

Study protocol

The protocol for the LabCyte assay was developed by J-TEC, and was based on the EpiSkin protocol (29). In the first phase of the study, the SOP (version 4.1) did not include the measurement of IL-1α release. At the second VMT meeting, in August 2008, the VMT discussed with the participating laboratories the results of the first phase and the content of the SOP, including the acceptance criteria for this validation study. Subsequent SOPs reflected the results of this discussion.

The second and third phases used different versions of the SOP, versions 5.0 and 6.1, respectively. A major difference between these versions was the elimination of the measurement of IL-1α release from version 6.1, in which the protocol specified that judgements on the classification had to be based on the LabCyte MTT assay alone. Other revisions were minor, and included changes in the

description of the formula for calculating viability, in the description of the use of a median of three runs for classification, and to the procedure for handling volatile substances. At a VMT meeting in July 2009, it was concluded that these revisions were minor, as long as the judgement for the classification was based on the MTT assay only. It was further decided that there was little difference between versions 5.0 and 6.1 of the SOP, apart from the elimination of the IL-1α release measurement.

LabCyte EPI-MODEL24 tissues were shipped from the supplier on Mondays and delivered to the recipients on Tuesdays. Upon receipt, the tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA), with 0.5ml per well of assay medium (which had the same composition as the culture medium), and incubated overnight (37°C, 5% v/v CO_2, humidified atmosphere). On the following day, the test chemicals were applied topically to the tissues. Liquids (25µl) were applied with a micropipette, and solids (25mg) were pre-moistened with 25µl sterile water before application. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied by using a micropipette with a cell saver-type tip. Each test chemical was applied to three tissues. In addition, 25µl of distilled water were added to three tissues, which served as the negative control, while three tissues were exposed to 25µl 5% w/v SLS as the positive control. After a 15-minute exposure, each tissue was carefully washed ten times with PBS (Invitrogen, Frederick, MD, USA) by using a wash bottle, to ensure the removal of any remaining test chemical from the surface. The washed tissues were then transferred to new 24-well plates, containing 1ml of fresh assay medium per well.

The test and control tissues were incubated for 42 hours (37°C, 5% CO_2, humidified atmosphere), then the conditioned medium was collected for determining the levels of IL-1α, and the washed tissues were transferred to new 24-well plates with 0.5ml of freshly prepared medium containing 1mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Dojindo Co., Kamimashiki, Kumamoto, Japan), for the MTT assay. The tissues were incubated for 3 hours (37°C, 5% CO_2, humidified atmosphere), then transferred to microtubes containing 0.3ml isopropanol, which completely immersed the tissues. Formazan extraction was performed at room temperature, and the tissues were allowed to stand overnight in the extraction solution. Subsequently, 200µl of the formazan-containing solutions were transferred to each well of a 96-well plate. The optical density of the samples was measured at 570nm with a reference wavelength of 650nm, and with isopropanol as a blank.

Tissue viability was calculated as a percentage, relative to the viability of the negative control, by using the equation: (see bottom of page)

The amount of IL-1α released into the conditioned medium after the 42-hour incubation was determined by using an IL-1α ELISA kit (Invitrogen), according to the manufacturer's instructions.

Prediction model of skin irritation

The acceptance criteria for the negative and positive controls, and the model for the prediction of skin irritation potential by using the LabCyte assay, were established to correspond to the equivalent conditions for EpiSkin, as described in the revised ECVAM performance standards (19). The acceptance criteria were:

1. a negative control OD greater than 0.7; and
2. a positive control viability lower than 40%.

The prediction model is described in Table 3. If the mean viability was equal to or less than 50%, no measurable amount of IL-1α was released.

The agreement between three independent viability measurements was used to indicate within-laboratory reproducibility, and the majority classification for each chemical was used to evaluate between-laboratory reproducibility.

The median value of three independent viability measurements was used to classify each chemical for the accuracy of the results. When only the tissue viability measurement was used for the classification, the classification was based entirely on the median of the three measurements. In the event that the three independent results from within an individual run were not in agreement, the mean of the two most similar results was used for the classification (i.e. minority classification), according to the prediction model.

In the third phase of the study, the criteria for classification were revised as follows:

- a substance is irritant if the median tissue viability is < 50%
- a substance is non-irritant if the median tissue viability is ≥ 50%

$$\text{Tissue viability (\%)} = \frac{[\text{Mean measured OD}_{\text{sample}}}{\text{Mean measured OD}_{\text{negative control}}]} \times 100$$

where measured OD = $[570\text{nm OD}_{\text{sample}} - 570\text{nm OD}_{\text{blank}}] - [650\text{nm OD}_{\text{sample}} - 650\text{nm OD}_{\text{blank}}]$.

Table 3: Positive criteria used for classification in the second phase of the study

Tissue viability (primary)	IL-1α ELISA (secondary)	Classification
Mean tissue viability ≤ 50%	—	Irritant
Mean tissue viability > 50%	Mean IL-1α release ≥ 120 pg/tissue	Irritant
Mean tissue viability > 50%	Mean IL-1α release < 120 pg/tissue	Non-irritant

IL-1α levels were not assessed, and IL-1α release was not used in the classification.

The independent biostatisticians had developed a standard Microsoft Excel® datasheet for use in the collection of data. The biostatisticians assembled all the submitted datasheets, decoded the chemical identifiers, and created the datasets that formed the basis of all the analyses. The data management procedures and statistical tools applied were approved by the VMT.

Quality assurance

All the participating laboratories worked according to the OECD GLP principles. The independent biostatisticians confirmed the authenticity of the data entered on the datasheet by comparing outputs from the spectrophotometers. Furthermore, JaCVAM assured the quality of all the documents and datasheets.

Results

Quality control (QC) of the tissue models

The QC data for the tissue models employed during this validation study, which are not shown, demonstrated that the OD after the MTT assay, and therefore the viability, of the negative control and after treatment for 18 hours with various concentrations of SLS solution was stable among the different batches provided to each laboratory. From these data, the VMT was able to confirm the completeness of the epithelial tissue layers used in this validation study. The participants did not need to perform any histological analysis after receiving LabCyte EPI-MODEL24, because J-TEC, the supplier of the tissues, had assessed the structure of all the batches prior to shipment. All the batches used for the validation study had passed the manufacturer's model supply criteria of LabCyte EPI-MODEL24.

First phase

In the first phase of the study, all the data for the negative and positive controls met the acceptance criteria, as shown in Tables 4 and 5, respectively. With the sole exception of ethanol, none of the chemicals tested in this phase of the study showed a significant variation in the triplicate tests performed at each laboratory (for Laboratory F, data for one run is missing; Table 5). The VMT judged cell viabilities from ethanol exposure at most laboratories to be around 50%, and although the data obtained from the different laboratories were similar, the within-laboratory variation was considerable. The experiments performed in this first phase indicated the transferability of the LabCyte MTT assay throughout the laboratories participating in the study. Based on the results obtained, the VMT decided that the transferability of this assay was high, and that all seven laboratories would participate in the subsequent phase of the study.

Table 4: Absorbance data obtained for the negative control used in the first phase of the study

Laboratory	Experiment 1 Absorbance	Experiment 2 Absorbance	Experiment 3 Absorbance	Mean	SD
A	1.073	0.928	1.007	1.003	0.073
B	0.930	1.245	1.042	1.072	0.160
C	0.960	0.869	0.761	0.863	0.100
D	0.987	0.928	0.939	0.951	0.031
E	0.840	0.884	0.973	0.899	0.068
F	1.049	0.934	0.968	0.984	0.059
G	1.147	1.159	1.074	1.127	0.046

Table 5: Tissue viability data obtained for the positive control and three chemicals used in the first phase of the study

Chemical	Laboratory	Exp. 1 Viability (%)	Exp. 2 Viability (%)	Exp. 3 Viability (%)	Mean	SD
Positive control (5% w/v sodium lauryl sulphate)	A	6.35	27.55	15.67	16.52	10.63
	B	3.94	3.51	3.97	3.81	0.26
	C	5.45	4.81	3.49	4.58	1.00
	D	11.74	7.22	14.08	11.02	3.49
	E	31.60	9.76	38.61	26.66	15.05
	F	3.10	2.89	2.93	2.97	0.11
	G	4.46	7.17	2.62	4.75	2.29
Ethanol	A	62.67	39.12	46.61	49.46	12.03
	B	41.08	50.86	86.58	59.51	23.95
	C	68.13	34.13	67.31	56.53	19.40
	D	68.57	40.52	33.03	47.37	18.73
	E	54.19	72.08	60.55	62.27	9.07
	F	ND	64.16	47.98	56.07	11.44
	G	4.68	5.23	6.67	5.53	1.03
Glycerol	A	103.63	104.17	98.48	102.09	3.14
	B	85.50	100.58	67.97	84.68	16.32
	C	101.24	99.41	104.84	101.83	2.76
	D	103.30	101.35	89.73	98.13	7.34
	E	101.75	98.06	99.04	99.62	1.91
	F	ND	97.23	96.00	96.62	0.87
	G	94.00	98.16	103.6	98.59	4.82
Naphthalene acetic acid	A	109.13	90.73	97.78	99.22	9.28
	B	93.96	103.91	103.96	100.61	5.76
	C	103.66	102.11	117.3	107.69	8.36
	D	102.28	98.15	94.56	98.33	3.86
	E	107.11	104.39	97.36	102.95	5.03
	F	ND	101.34	102.07	101.7	0.52
	G	92.20	101.04	105.52	99.59	6.78

Exp. = experiment, ND = No data.

Second and third phases

One of the limitations of this study was that the experiments could not be performed under full GLP compliance, because some of the participating laboratories were not in GLP-compliant facilities. However, the VMT conducted the experiments according to GLP principles, and all the submitted datasheets and documentation sheets were verified.

There were a few comments from each laboratory in the document sheets; for instance: 'the application of coded chemical No. 21 (cinnamaldehyde) caused the cups to become discoloured and crystallised'; and 'the application of coded chemical No. 23 (potassium hydroxide 5% w/v) caused the model's layers to become desquamated'.

With regard to the absorbance (i.e. mean OD) of the negative control for each run, in Laboratory A, the mean OD for run 1 was 0.59 (0.61, 0.58 and 0.57) in the second phase of the study. The VMT did not accept this result under the acceptance criteria and, instead, accepted the results of tests 2–4 from Laboratory A (runs 1, 2 and 3, respectively). The reason for this was unclear (Data not shown).

Excluding these data, all the negative control data from each laboratory involved in the study satisfied the pre-specified acceptance criteria, as shown in Table 4 for phase 1 (data for the second and third phases are not shown). Therefore, for this assay, the rate of invalid tests was 1/508 or 0.2% — i.e. 400 data points (3 runs × 7 laboratories × 19 chemicals + 1 run) in the second phase of the study, plus 108 data points (3 runs × 6 labs × 6 chemicals) in the third phase study.

Given the results of three independent cell viability readings, and the summary statistics for the positive control at each laboratory, the data were sufficient for the positive control to meet the acceptance criteria. The distribution of the data for cell viability after treatment with chemical No. 13 (5% w/v SLS, i.e. the same chemical as used as the positive control) showed high repeatability and reproducibility in the second and third phases of the study (Tables 6 and 7, respectively). The sum-

Table 6: Tissue viability data obtained for the positive control and chemical No. 13 in the second phase of the study

Chemical	Laboratory	Experiment 1 Viability (%)	Experiment 2 Viability (%)	Experiment 3 Viability (%)	Mean	SD
No. 13*	A	12.2	3.6	2.2	6.0	4.4
	B	5.2	3.2	12.5	7.0	4.0
	C	9.9	5.0	3.3	6.1	2.8
	D	3.8	3.6	2.5	3.3	0.6
	E	12.9	6.7	4.7	8.1	3.5
	F	12.0	3.1	7.4	7.5	3.6
	G	10.7	8.0	3.3	7.3	3.1
Positive control	A	5.9	8.8	2.5	5.7	2.6
	B	5.2	12.3	7.8	8.4	2.9
	C	4.1	5.4	3.8	4.4	0.7
	D	5.7	2.6	3.3	3.9	1.3
	E	4.1	12.6	5.6	7.4	3.7
	F	3.5	2.9	3.2	3.2	0.2
	G	3.1	10.8	4.2	6.0	3.4

**Chemical No. 13 is 5% w/v sodium lauryl sulphate, which replaced the tri-isobutyl phosphate in the second phase of the study and which was also used as the positive control.*

mary statistics for tissue viability data for each chemical in the second and third phases are shown in Table 8. As mentioned above, all the data obtained were valid.

The classifications based on the median of three independent cell viability results (i.e. taking into consideration data from the MTT assay only) are shown in Table 9. In the second phase of the study, the levels of IL-1α release were measured in those cases where the mean tissue viability was > 50% (Table 10). As shown in Tables 10 and 11, IL-1α results altered the classification of three chemicals. The classification of chemical No. 5 (allyl phenoxyacetate) by Laboratory F was changed, leading to its misclassification as a false positive result. In contrast, the classification of the other two chemicals had been underestimated, and they subsequently were placed in the correct category. Thus, while the use of IL-1α release data was responsible for changing the classification of a few chemicals, in general, it did not have a major effect. That is, the determination of IL-1α release did not significantly contribute to the performance of the assay.

The VMT therefore decided that the following analysis should be performed only on tissue viability data (by using the MTT assay). With regard to the within-laboratory reproducibility of the LabCyte MTT assay, some disagreements occurred between measurements: discrepancies were detected between three triplicate viability measurements in Laboratories A and F, two in Laboratories B and D, and one in Laboratory E (none occurred in Laboratories C or G; data not shown). Of the total of 170 tests performed (on 25 chemicals in six laboratories, plus 20 chemicals in one laboratory), there were 11 (6.5%) discrepancies, resulting in a within-laboratory reproducibility ratio of 93.5%. Therefore, the VMT concluded that this assay showed high within-laboratory

Table 7: Tissue viability data obtained for the positive control in the third phase of the study

	Laboratory	Experiment 1 Viability (%)	Experiment 2 Viability (%)	Experiment 3 Viability (%)	Mean	SD
Positive control*	A	6.4	2.2	1.8	3.5	2.6
	B	9.4	2.3	1.7	4.4	4.3
	C	8.2	7.3	2.4	6.0	3.1
	D	3.5	2.5	2.1	2.7	0.7
	F	8.5	4.1	2.7	5.1	3.0
	G	11.7	2.5	3.3	5.8	5.1

** The positive control was 5% w/v sodium lauryl sulphate.*

Table 8: A summary of the tissue viability data obtained for each chemical in the second and third phases of the study

No.	Statistics	Laboratory A	B	C	D	E	F	G
a) In the second phase								
1	Mean	17.9	24.5	14.4	11.0	31.9	12.0	11.7
	SD	11.3	19.8	5.2	2.9	6.4	2.0	2.0
	Min	11.2	10.4	10.6	9.1	25.2	10.4	10.6
	Max	31.0	47.1	20.3	14.3	38.1	14.3	14.0
2	Mean	73.8	72.4	87.8	86.6	88.0	72.7	98.0
	SD	7.7	14.3	1.9	17.6	12.8	4.8	4.6
	Min	65.2	61.7	85.8	67.6	76.4	67.2	94.8
	Max	79.8	88.7	89.7	102.3	101.8	75.7	103.3
3	Mean	104.7	98.5	94.5	106.4	113.3	94.8	101.7
	SD	4.1	4.6	1.3	5.8	14.1	2.7	8.9
	Min	100.9	93.3	93.1	101.4	103.9	92.5	93.4
	Max	109.1	102.3	95.7	112.8	129.6	97.9	111.1
4	Mean	99.3	97.8	98.2	101.8	115.3	95.2	105.9
	SD	6.1	3.0	1.5	3.9	11.0	4.2	3.4
	Min	95.2	94.4	97.1	98.4	105.2	92.7	103.3
	Max	106.3	100.2	99.9	106.1	127.1	100.1	109.8
5	Mean	77.0	72.7	91.9	72.0	94.3	55.4	91.7
	SD	2.5	11.4	3.1	6.8	7.6	16.3	4.5
	Min	74.1	61.7	89.2	66.1	89.6	39.3	88.4
	Max	78.5	84.5	95.2	79.4	103.0	71.9	96.8
6	Mean	84.8	80.7	81.2	92.1	89.7	87.8	74.2
	SD	6.9	2.8	2.3	9.9	7.8	6.7	17.7
	Min	79.4	77.9	79.1	82.7	81.5	81.1	54.1
	Max	92.5	83.5	83.6	102.4	97.0	94.4	87.2
7	Mean	18.2	12.2	17.4	18.4	20.3	20.8	24.6
	SD	5.7	1.3	3.0	4.2	2.5	8.9	6.1
	Min	12.6	10.8	15.2	13.8	17.5	15.6	19.9
	Max	24.1	13.2	20.8	21.7	22.2	31.1	31.5
8	Mean	99.1	97.4	78.3	106.6	105.6	94.7	103.7
	SD	11.4	9.5	3.6	3.3	8.1	4.4	4.6
	Min	90.2	86.7	75.3	103.0	100.9	89.7	100.9
	Max	111.9	104.8	82.3	109.4	114.9	97.8	109.0
9	Mean	103.7	105.4	98.9	102.2	109.4	94.3	105.9
	SD	8.2	7.6	6.8	3.6	6.0	6.8	5.4
	Min	97.1	96.7	93.5	98.1	103.9	86.5	102.3
	Max	112.8	110.1	106.6	105.0	115.8	98.8	112.1
10	Mean	102.1	112.2	104.9	109.3	118.8	102.1	109.8
	SD	14.8	2.8	2.2	4.5	12.3	1.6	2.9
	Min	86.5	110.1	103.6	105.5	107.5	101.2	107.9
	Max	115.9	115.4	107.5	114.3	132.0	104.0	113.1
11	Mean	108.1	105.1	96.6	103.6	113.0	98.6	104.1
	SD	8.7	1.4	3.8	1.9	9.0	4.3	4.4
	Min	98.1	103.7	94.1	102.4	105.5	94.6	100.5
	Max	113.7	106.6	101.0	105.8	123.1	103.1	109.0
12	Mean	20.7	21.7	32.6	52.6	56.9	52.8	99.5
	SD	6.7	5.0	10.7	24.4	1.1	22.2	12.5
	Min	15.3	15.9	24.9	27.4	55.6	27.2	87.7
	Max	28.2	24.6	44.8	76.2	57.8	66.0	112.6

Table 8: continued

No.	Statistics	Laboratory						
		A	B	C	D	E	F	G
a) In the second phase								
13	Mean	6.0	7.0	6.1	3.3	8.1	7.5	7.3
	SD	5.4	4.9	3.4	0.7	4.3	4.5	3.7
	Min	2.2	3.2	3.3	2.5	4.7	3.1	3.3
	Max	12.2	12.5	9.9	3.8	12.9	12.0	10.7
14	Mean	8.2	9.7	11.1	10.9	12.2	15.6	12.0
	SD	2.6	2.1	3.1	0.8	1.9	2.2	1.4
	Min	6.6	8.3	9.1	10.2	10.4	13.1	10.6
	Max	11.1	12.1	14.7	11.7	14.2	17.0	13.5
15	Mean	8.8	9.8	13.5	8.6	10.3	7.2	14.4
	SD	2.1	0.4	5.6	0.6	1.4	1.4	9.0
	Min	7.1	9.3	8.1	8.0	8.7	5.9	9.2
	Max	11.1	10.2	19.3	9.2	11.3	8.6	24.7
16	Mean	53.3	81.4	73.1	45.0	89.1	59.1	77.0
	SD	18.7	23.9	18.9	31.2	9.8	8.6	11.0
	Min	32.2	54.1	51.5	18.1	78.7	50.4	64.9
	Max	67.9	98.3	86.3	79.2	98.2	67.5	86.5
17	Mean	5.5	4.9	5.8	5.3	6.9	5.6	5.6
	SD	0.7	0.6	0.4	1.4	1.8	1.2	0.5
	Min	4.8	4.5	5.3	3.9	5.4	4.5	5.3
	Max	6.1	5.7	6.0	6.6	8.9	6.9	6.2
18	Mean	61.9	65.7	88.7	70.9	85.1	73.9	90.9
	SD	31.7	29.8	2.2	12.4	15.0	6.0	4.5
	Min	25.3	46.5	87.3	59.0	69.1	69.2	85.9
	Max	82.1	100.0	91.2	83.7	98.9	80.6	94.4
19	Mean	28.7	39.2	22.5	25.6	59.5	66.3	39.8
	SD	19.6	32.4	12.5	6.5	29.7	23.9	4.5
	Min	15.0	10.9	10.0	18.2	26.1	40.1	35.8
	Max	51.1	74.6	35.0	30.4	83.1	87.0	44.7
20	Mean	23.3	14.0	8.6	19.2	8.4	8.0	8.1
	SD	12.1	9.3	1.6	10.8	2.3	0.2	1.2
	Min	9.3	8.0	7.6	9.6	6.2	7.8	6.7
	Max	31.1	24.8	10.4	30.9	10.7	8.2	8.8
b) In the third phase								
21	Mean	13.8	11.0	16.0	12.3	—	12.3	12.2
	SD	14.0	11.1	13.2	13.2	—	11.4	13.7
	Min	13.3	10.2	12.3	9.9	—	11.3	8.7
	Max	14.2	11.8	22.5	13.8	—	14.3	14.3
22	Mean	2.0	2.3	2.8	3.6	—	2.5	4.1
	SD	1.5	2.2	2.9	3.9	—	2.6	3.9
	Min	1.5	2.2	2.5	3.0	—	1.7	3.7
	Max	3.1	2.5	3.0	4.0	—	3.2	4.7
23	Mean	0.8	0.8	1.0	3.2	—	2.2	0.6
	SD	0.7	0.8	1.0	2.0	—	1.0	0.4
	Min	0.5	0.7	0.7	0.8	—	0.8	0.3
	Max	1.3	1.1	1.4	6.9	—	4.8	1.0
24	Mean	15.6	18.5	12.6	17.2	—	13.9	16.2
	SD	14.5	16.0	12.6	18.3	—	13.8	15.2
	Min	13.6	15.5	12.5	10.3	—	8.8	14.1
	Max	18.6	24.0	12.7	23.0	—	19.2	19.3

Table 8: continued

No.	Statistics	Laboratory A	B	C	D	E	F	G
b) In the third phase								
25	Mean	3.4	3.2	3.4	5.3	—	4.2	4.1
	SD	3.9	3.4	3.4	3.9	—	4.2	4.1
	Min	1.8	2.7	3.3	3.7	—	3.2	3.4
	Max	4.5	3.5	3.5	8.2	—	5.0	5.1
26	Mean	5.5	5.8	6.6	5.7	—	5.9	7.2
	SD	5.6	6.1	6.5	5.4	—	5.2	7.2
	Min	5.4	4.2	6.5	5.4	—	5.0	6.8
	Max	5.7	7.2	6.8	6.4	—	7.4	7.6

'—' indicates that the data are not available from this laboratory.

Table 9: The classification obtained by using three independent tissue viability results from the second and third phases of the study

No.	GHS label	*In vivo* score	Laboratory A	B	C	D	E	F	G
1	No category	0.0	+ve	+ve	+ve	+ve	+ve	+ve	+ve
2	No category	0.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
3	No category	0.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
4	No category	0.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
5	No category	0.3	–ve	–ve	–ve	–ve	–ve	–ve	–ve
6	No category	0.3	–ve	–ve	–ve	–ve	–ve	–ve	–ve
7	No category	1.0	+ve	+ve	+ve	+ve	+ve	+ve	+ve
8	No category	1.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
9	No category	1.7	–ve	–ve	–ve	–ve	–ve	–ve	–ve
10	No category	1.7	–ve	–ve	–ve	–ve	–ve	–ve	–ve
11	No category	2.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
12	No category	2.0	**+ve**	**+ve**	**+ve**	–ve	–ve	–ve	–ve
21	No category	2.0	+ve	+ve	+ve	+ve	+ve	+ve	+ve
14	Category 2	2.3	+ve	+ve	+ve	+ve	—	+ve	+ve
15	Category 2	2.3	+ve	+ve	+ve	+ve	+ve	+ve	+ve
16	Category 2	2.7	–ve	–ve	–ve	**+ve**	–ve	–ve	–ve
17	Category 2	2.7	+ve	+ve	+ve	+ve	+ve	+ve	+ve
22	Category 2	2.7	+ve	+ve	+ve	+ve	—	+ve	+ve
23	Category 2	2.7	+ve	+ve	+ve	+ve	—	+ve	+ve
18	Category 2	3.0	–ve	–ve	–ve	–ve	–ve	–ve	–ve
19	Category 2	3.0	+ve	+ve	+ve	+ve	**–ve**	**–ve**	+ve
24	Category 2	3.3	+ve	+ve	+ve	+ve	—	+ve	+ve
25	Category 2	3.3	+ve	+ve	+ve	+ve	—	+ve	+ve
26	Category 2	4.0	+ve	+ve	+ve	+ve	—	+ve	+ve
20	Category 2	4.0	+ve	+ve	+ve	+ve	+ve	+ve	+ve

— = Not tested. Bold type indicates minority classification. +ve = positive (irritant); –ve = negative (non-irritant).

Table 10: An overview of the levels of IL-1α release measured in each laboratory

No.	GHS label	*In vivo* score	Experiment	Laboratory						
				A	B	C	D	E	F	G
1	No category	0	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—
2	No category	0	1	132.8*	52.9	59.3	41.2	60.7	61.3	9.4
			2	68.1	56.5	37.0	89.1	68.4	99.3	9.6
			3	97.6	41.1	76.0	72.4	46.0	70.1	12.6
3	No category	0	1	12.0	9.5	15.5	8.6	23.2	12.7	8.1
			2	7.1	8.6	11.7	19.9	10.5	9.2	11.9
			3	10.7	10.3	12.9	9.4	11.3	6.7	15.7
4	No category	0	1	10.0	6.0	8.0	11.7	9.5	2.5	6.3
			2	5.3	8.0	5.5	13.2	15.1	2.6	8.6
			3	6.3	4.7	7.2	7.9	9.7	3.4	6.8
5	No category	0.3	1	122.0*	97.6	24.3	81.2	57.7	**183.5***	15.4
			2	35.7	63.5	35.1	115.3	36.6	—	28.5
			3	44.4	26	31.2	49.4	33.0	**191.6***	33.2
6	No category	0.3	1	59.0	85.7	114.0	85.6	94.4	60.8	112.5
			2	62.9	93.6	104.9	139.5*	81.4	48.1	62.1
			3	68.8	85.1	82.9	64.5	52.9	54.8	147.1
7	No category	1	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—
8	No category	1	1	8.2	9.4	84.1	4.1	6.9	21.4	5.3
			2	3.6	6.4	31.6	10.4	8.5	4.9	5.8
			3	6.0	4.1	33.1	5.2	6.7	2.1	7.2
9	No category	1.7	1	10.9	17.1	11.2	42.6	29.5	33.0	7.4
			2	19.8	8.8	8.8	32.2	6.5	25.3	9.7
			3	31.3	6.8	20.1	21.3	11.2	24.7	10.6
10	No category	1.7	1	27.9	7.4	31.3	41.2	46.5	39.3	9.8
			2	17.1	12.7	15.0	50.4	26.7	26.7	14.5
			3	66.2	12.2	30.0	42.1	26.3	24.2	13.2
11	No category	2	1	5.0	31.1	18.0	15.3	10.4	16.2	6.4
			2	3.3	11.9	15.8	19.0	9.7	8.1	7.5
			3	18.2	5.0	8.9	8.7	8.6	12.6	11.9
12	No category	2	1	—	—	—	157.2	120.4	—	34.5
			2	—	—	—	113	118.6	90.2	27.3
			3	—	—	—	—	58.3	66.2	13.6
14	Category 2	2.3	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—
15	Category 2	2.3	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—
16	Category 2	2.7	1	**86.9***	68.1	129.4	—	126.8	116.5	90.8
			2	—	100.2	74.4	169.7	76.1	107.5	70.9
			3	**121.2***	42.5	83.6	—	73.1	87.3	79.2

**The mean IL-1α release ≥ 120pg/tissue; — = not tested.*
The values in bold refer to cases where IL-1α release data changed the classification assigned by using the MTT assay data.

Table 10: continued

No.	GHS label	*In vivo* score	Experiment	Laboratory						
				A	B	C	D	E	F	G
17	Category 2	2.7	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—
18	Category 2	3	1	61.5	—	60.6	**90.3**	86.9	114.5	18.0
			2	57.7	104.9	45.8	**221.3***	98.7	76.4	45.1
			3	—	17.2	51.4	**138.1***	63.9	102.2	22.1
19	Category 2	3	1	—	57.3	—	—	109.2		
			2	—	—	—	—	—	69.2	
			3	102.3	—	—	—	68.0	59.5	
20	Category 2	4	1	—	—	—	—	—	—	—
			2	—	—	—	—	—	—	—
			3	—	—	—	—	—	—	—

**The mean IL-1α release ≥ 120pg/tissue; — = not tested.*
The values in bold refer to cases where IL-1α release data changed the classification assigned by using the MTT assay data.

reproducibility. For the between-laboratory reproducibility of the LabCyte MTT assay, the minority classifications were for six results (chemical No. 12: three results; chemical No. 16: one result; and chemical No. 19: two results) among all the 170 classifications, as shown in Table 9. This corresponded to only 3.5% of the total, so the between-laboratory reproducibility was 96.5%. Based on these results, the VMT decided this assay showed high between-laboratory reproducibility.

For the evaluation of accuracy, the data for chemical No. 13 were excluded, because this chemical was not included in the lists within the original ECVAM performance standards (18) or in the ESAC statements (5, 6). The sensitivity, specificity and accuracy of the MTT assay (in relation to the UN GHS category) for the 25 chemicals tested in the second and third phases of the study are shown in Table 12. Collectively, the sensitivity, specificity and accuracy of this prediction model at the different laboratories varied between 75–91.6%, 69.2–76.9% and 76–84%, respectively. Similar values were obtained within each laboratory. The mean prediction values of the LabCyte MTT assay were: sensitivity 83.3%, specificity 73.1%, and accuracy 78.0% (Table 13). The ESAC statement proposed that the performance of a skin irritation model should be as follows: sensitivity 80%, specificity 70% and accuracy 75% (18, 19), so this requirement was fully satisfied.

Discussion

This validation study refers to the original, or the revised, ECVAM performance standards (18, 19). While the study was being conducted, the draft OECD performance standards, which were based on the ECVAM performance standards, were the subject of discussion by OECD international skin irritation experts. Therefore, we were unable to follow current OECD test guidelines for the calculation of within-laboratory and between-laboratory reproducibility, or during the data analysis (22).

Table 11: The chemicals that had their classification changed as a result of IL-1α release data

No.	Name	CAS number	GHS label	*In vivo* score	Laboratory	Classification (MTT assay)	Classification (MTT and IL-1α release)
5	Allyl phenoxyacetate	7493-74-5	No category	0.3	F	–ve	+ve
16	1-Bromohexane	111-25-1	Category 2	2.7	A	–ve	+ve
18	di-*n*-Propyl disulphide	629-19-6	Category 2	3.0	D	–ve	+ve

+ve = positive; –ve = negative.

Table 12: A comparison of the classification based on the LabCyte MTT assay data with the actual GHS classifications, in the second and third phases of the study

Index	Laboratory					
	A	B	C	D	F	G
Sensitivity	10/12 83.3%	10/12 83.3%	10/12 83.3%	11/12 91.6%	9/12 75%	10/12 83.3%
Specificity	9/13 69.2%	9/13 69.2%	9/13 69.2%	10/13 76.9%	10/13 76.9%	10/13 76.9%
Accuracy	19/25 76%	19/25 76%	19/25 76%	21/25 84%	19/25 76%	20/25 80%

A total of 25 substances were tested.

Reliability

All the negative control data from the LabCyte MTT assay showed high repeatability, as well as within-laboratory and between-laboratory reproducibility (data for the first phase is shown in Table 4). The same was true for the positive control data — high repeatability, within-laboratory and between-laboratory reproducibility were reported throughout the study (Tables 5–7).

When we conducted this study, we referred to the original ECVAM performance standards (18). In this document, one of the defined acceptance criteria was that the data range should have a standard deviation ≤ 18%. After the first phase of the study, the VMT discussed the topic in relation to the LabCyte assay. Ultimately, the VMT decided that this particular criterion (i.e. standard deviation ≤ 18%) was not appropriate, because, at the time, the amount of data was not enough to set this kind of range. Instead of this criterion, the VMT set another indicator of within-laboratory reproducibility — the agreement between three independent viability measurements. In order to evaluate between-laboratory reproducibility, majority classifications for each chemical was used. As a result, the ratio of within-laboratory reproducibility was 93.5%, and for between-laboratory reproducibility it was 96.5%. Based on these values, the VMT decided that the Labcyte MTT assay showed high within-laboratory and between-laboratory reproducibility. As shown in the next section, the reliability of the model was high. Therefore, the criteria with regard to the range may not be required for this *in vitro* tissue model, even though the variation should be assessed.

For three of the test chemicals, the classifications based on the LabCyte MTT assay were not consistent among all seven laboratories. These chemicals were No. 12 (terpinyl acetate), No. 16 (1-bromohexane) and No. 19 (butyl methacrylate), as shown in Table 9. We consider this a peculiarity of viability measurements close to the threshold, since when chemical treatment results in a tissue viability of about 50%, laboratory-dependent discrepancies might occur. Chemicals that elicit this sort of response might not be suitable for this type of validation study. It should be noted that chemicals No. 12 and No. 19 have been eliminated from the list of chemicals in the revised ECVAM performance standards (19).

On the other hand, the other 22 chemicals exhibited consistent classifications between the laboratories (Table 9), while chemical No. 18 (di-*n*-propyl

Table 13: The mean and range of sensitivity, specificity and accuracy of the LabCyte MTT assay classifications *versus* UN GHS classifications, in the second and third phases of the study

	n	Mean	Minimum	Maximum	ECVAM criterion
Sensitivity (%)	6	83.3	75.0	91.6	80.0
Specificity (%)	6	73.1	69.2	76.9	70.0
Accuracy (%)	6	78.0	76.0	84.0	75.0

A total of 25 substances were tested.

disulphide) exhibited large variation. These results indicate that the model is highly reliable.

Predictivity

The mean sensitivity, specificity and accuracy of this prediction model are 83.3%, 73.1% and 78.0%, respectively (Table 13). The ESAC statement proposed that the performance of a skin irritation model should be as follows: sensitivity 80%, specificity 70% and accuracy 75% (18, 19). Therefore, our results satisfied these values.

The VMT detected four false positives: chemicals No. 1 (1-bromo-4-chlorobutane), No. 7 (4-methylthio-benzaldehyde), No. 12 (terpinyl acetate) and No. 21 (cinnamaldehyde) were among the 13 chemicals classified as non-irritant. Furthermore, there were three false negatives: chemicals No. 16 (1-bromohexane), No. 18 (di-*n*-propyl disulphide) and No. 19 (butyl methacrylate), which were among the 12 chemicals classified as irritant. In order for this model to be of use in regulatory assessment, it is important to determine the causes of false-negative or false-positive results. Although chemical No. 18 is an irritant in rabbits, it is a non-irritant chemical with human tissue. We propose that chemical No. 18, like chemical No. 16, was scored as a false negative because its viability was around 50%, which caused different results to be obtained in different laboratories for these two coded chemicals.

The necessity of IL-1α release measurement

IL-1α is a cytokine produced by keratinocytes, and is a well-known irritation marker. It is a key player when the mode of action of skin irritation is considered (30). Previously, Coquette *et al.* reported that the upregulation of IL-1α mRNA was observed after topical application of sensitisers and irritants, but only the latter significantly increased extracellular IL-1α (31). The determination of IL-1α release levels, in association with MTT conversion to formazan, is necessary to discriminate and classify between irritant and sensitising agents in a single assay, and thus represents a potential *in vitro* alternative to two classical *in vivo* assays. Spielmann *et al.* (16) reported the necessity of these endpoints with EpiSkin. For the MTT assay only, the sensitivity was 75% and the specificity was 81%. When the MTT and IL-1α release assays were combined, the sensitivity increased to 91% and the specificity was 79% (16). The ESAC has also made recommendations on the same combination of assays, MTT and IL-1α release, when using EpiSkin (6).

In contrast, our data show that IL-1α release determination changed the classification only for chemicals No. 5 (allyl phenoxyacetate) in Laboratory F, No. 16 (1-bromohexane) in Laboratory A and No. 18 (di-*n*-propyl disulphide) in Laboratory D, as shown in Tables 10 and 11. The effect of IL-1α release data on the reliability of these results was small. Therefore, the VMT considered the IL-1α release measurement in the LabCyte assay to be unnecessary for this protocol, although additional validation studies involving IL-1α release will be required to confirm this decision. Spielmann *et al.* have also reported the sensitivity of the EpiDerm assay (MTT data only) to be 57% and the specificity 85%, while the predictive capacity of the EpiDerm assay did not improve by the measurement of IL-1α release (16). In addition, OECD Test Guideline No. 439 has not approved the use of IL-1α release determination in the *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method (22).

Conclusion

The LabCyte MTT assay demonstrated high reliability, both within-laboratory and between-laboratory, and good reliability for the positive control (100%). In addition, the data showed acceptable relevance (77.5% overall accuracy, 82.3% overall sensitivity and 72.6% overall specificity) by using solely the MTT assay. Therefore, we found the assay to be suitable for use as a stand-alone method to distinguish between skin irritants and non-irritants. However, these results were based on, at most, 25 chemicals. Since this model demonstrated high reliability, we plan further investigations with additional chemicals, which will be conducted by the lead laboratory.

Acknowledgements

This validation study was supported by the JSAAE (Japanese Society for the Alternative to Animal Experiments) and a Health and Labour Sciences Research Grant, from the WHLW (Ministry of Health, Labour and Welfare), Japan.

Received 24.05.11; received in final form 22.12.11; accepted for publication 05.01.12.

References

1. Draize, J.H., Woodard, G. & Calvery, H.O. (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *Journal of Pharmacology & Experimental Therapeutics* **82**, 377–390.
2. OECD (2002). *OECD Guideline for the Testing of Chemicals. No. 404. Acute Dermal Irritation/ Corrosion*, 13pp. Paris, France: Organisation for

Economic Co-operation and Development. Available at: http://www.oecd-ilibrary.org/docserver/download/fulltext/9740401e.pdf?expires=1326212784&id=id&accname=freeContent&checksum=479E6FD0B64BAE98FE63C079EB226F82 (Accessed 10.01.12).
3. Cannon, C.L., Neal, P.J., Southee, J.A., Kubilus, J. & Klausner, M. (1994). New epidermal model for dermal irritancy testing. *Toxicology in Vitro* **8**, 889–891.
4. Ponec, M., Boelsma, E., Weerheim, A., Mulder, A., Bouwstra, J. & Mommaas, M. (2000). Lipid and ultrastructural characterization of reconstructed skin models. *International Journal of Pharmaceutics* **203**, 211–225.
5. ECVAM (2007). *Statement on the Validity of* In-vitro *Tests for Skin Irritation*, 7pp. Ispra, Italy: European Commission, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods, Scientific Advisory Committee. Available at: http://iccvam.niehs.nih.gov/methods/dermal/epiderm/ESAC26-SkinIrritation.pdf (Accessed 10.01.12).
6. ECVAM (2008). *Statement on the Scientific Validity of In-vitro Tests for Skin Irritation Testing*, 7pp. Ispra, Italy: European Commission, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods, Scientific Advisory Committee. Available at: http://ecvam.jrc.ec.europa.eu/ (Accessed 10.01.12).
7. ECVAM (2009). *Statement on the Performance Under UN GHS of Three* In-vitro *Assays for Skin Irritation Testing and the Adaptation of the Reference Chemicals and Defined Accuracy Values of the ECVAM Skin Irritation Performance Standards*, 8pp. Ispra, Italy: European Commission, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods, Scientific Advisory Committee. Available at http://ecvam.jrc.ec.europa.eu/ (Accessed 10.01.12).
8. UN (2007). *Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Second Revised Edition*, 555pp. Geneva, Switzerland: United Nations. Available at: http://www.unece.org/fileadmin/DAM/trans/danger/publi/ghs/ghs_rev02/English/00e_intro.pdf (Accessed 11.01.12).
9. Fentem, J.H., Briggs, D., Chesné, C., Elliott, G.R., Harbell, J.W., Heylings, J.R., Portes, P., Roguet, R., van de Sandt, J.J. & Botham, P.A. (2001). A prevalidation study on *in vitro* tests for acute skin irritation. Results and evaluation by the Management Team. *Toxicology in Vitro* **15**, 57–93.
10. Portes, P., Grandidier, M-H., Cohen, C. & Roguet, R. (2002). Refinement of the Episkin protocol for the assessment of acute skin irritation of chemicals: Follow-up to the ECVAM prevalidation study. *Toxicology in Vitro* **16**, 765–770.
11. Zuang, V., Balls, M., Botham, P.A., Coquette, A., Corsini, E., Curren, R.D., Elliott, G.R., Fentem, J.H., Heylings, J.R., Liebsch, M., Medina, J., Roguet, R., van de Sandt, J.J., Wiemann, C. & Worth, A.P. (2002). Follow-up to the ECVAM prevalidation study on *in vitro* tests for acute skin irritation. The European Centre for the Validation of Alternative Methods Skin Irritation Task Force report 2. *ATLA* **30**, 109–129.
12. Kandárová, H., Liebsch, M., Genschow, E., Gerner, I., Traue, D., Slawik, B. & Spielmann, H. (2004). Optimisation of the EpiDerm test protocol for the upcoming ECVAM validation study on *in vitro* skin irritation tests. *ALTEX* **21**, 107–114.
13. Kandárová, H., Liebsch, M., Gerner, I., Schmidt, E., Genschow, E., Traue, D. & Spielmann, H. (2005). The EpiDerm test protocol for the upcoming ECVAM validation study on *in vitro* skin irritation tests — An assessment of the performance of the optimised test. *ATLA* **33**, 351–367.
14. Cotovio, J., Grandidier, M-H., Portes, P., Roguet, R. & Rubinstenn, G. (2005). The *in vitro* acute skin irritation of chemicals: Optimisation of the EPISKIN prediction model within the framework of the ECVAM validation process. *ATLA* **33**, 329–349.
15. Hoffmann, S. (2006). *ECVAM Skin Irritation Validation Study Phase II: Analysis of the primary endpoint MTT and the secondary endpoint IL-1α*, 190pp. Ispra, Italy: European Commission, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods. Available at: http://ecvam.jrc.it/ft_doc/ATT00042_s.pdf (Accessed 11.01.12).
16. Spielmann, H., Hoffmann, S., Liebsch, M., Botham, P., Fentem, J.H., Eskes, C., Roguet, R., Cotovio, J., Cole, T., Worth, A., Heylings, J., Jones, P., Robles, C., Kandárová, H., Gamer, A., Remmele, M., Curren, R., Raabe, H., Cockshott, A., Gerner, I. & Zuang, V. (2007). The ECVAM international validation study on *in vitro* tests for acute skin irritation: Report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test. *ATLA* **35**, 559–601.
17. Eskes, C., Cole, T., Hoffmann, S., Worth, A., Cockshott, A., Gerner, I. & Zuang, V. (2007) .The ECVAM international validation study on *in vitro* tests for acute skin irritation: Selection of test chemicals. *ATLA* **35**, 603–619.
18. Griesinger, C., Barroso, J., Zuan, V., Cole, T., Genschow, E. & Liebsch, M. (2010). *Explanatory Background Document to the OECD Draft Test Guideline on* In Vitro *Skin Irritation Testing*, 209pp. Ispra, Italy: European Commission, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods.
19. ECVAM (2009). *ESAC Statement on the Performance Standards (PS) for* In Vitro *Skin Irritation Testing Using Reconstructed Human Epidermis*, 7pp. Ispra, Italy: European Commission, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods, Scientific Advisory Committee Available at: http://ecvam.jrc.it/publication/ESAC31_statement_updated-skin-irritation-PS_200909-22.pdf (Accessed 20.01.12).
20. ECH (2010). *European Chemicals Agency*. Helsinki, Finland: European Chemicals Agency. Available at: http://ec.europa.eu/echa/home_en.html (Accessed 23.01.12).
21. Anon. (2004). *Commission Staff Working Document. Timetables for the Phasing-out of Animal Testing in the Framework of the 7th Amendment to the Cosmetics Directive (*Council Directive 76/768/EEC*)*, 8pp. Brussels, Belgium: Commission of the European Communities. Available at: http://ec.europa.eu/consumers/sectors/cosmetics/files/doc/antest/sec_2004_1210_en.pdf (Accessed 23.01.12).
22. OECD (2010). *OECD Guideline for the Testing of*

Chemicals. No. 439. In Vitro *Skin Irritation: Reconstructed Human Epidermis Test Method*, 18pp. Paris, France: Organisation for Economic Co-operation and Development. Available at: http://www.oecd-ilibrary.org/docserver/download/fulltext/9743901e.pdf?expires=1326721637&id=id&accname=freeContent&checksum=DE7DC6821D95D80CB715E855076DACF8 (Accessed 16.01.12).
23. Katoh, M., Hamajima, F., Ogasawara, T. & Hata, K-I. (2009). Assessment of the human epidermal model LabCyte EPI-MODEL for *in vitro* skin irritation testing according to European Centre for the Validation of Alternative Methods (ECVAM)-validated protocol. *Journal of Toxicological Science* **34**, 327–334.
24. OECD (2005). *OECD Series on Testing and Assessment. No. 34. Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment*, 96pp. Paris, France: Organisation for Economic Co-operation and Development. Available at: http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2005)14&doclanguage=en (Accessed 16.01.12).
25. Hartung, T., Bremer, S., Casati, S., Coecke, S., Corvi, R., Fortaner, S., Gribaldo, L., Halder, M., Hoffmann, S., Roi, A.J., Prieto, P., Sabbioni, E., Scott, L., Worth, A. & Zuang, V. (2004). A modular approach to the ECVAM principles on test validity. *ATLA* **32**, 467–472.
26. Rheinwald, J.G. & Green, H. (1975). Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. *Cell* **6**, 331–344.
27. Green, H. (1978). Cyclic AMP in relation to proliferation of the epidermal cell: New view. *Cell* **15**, 801–811.
28. OECD (1999). *OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring. No 5. Compliance of Laboratory Suppliers with GLP Principles*, 8pp. Paris, France: Organisation for Economic Co-operation and Development. Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(99)21&docLanguage=En (Accessed 16.01.12).
29. ECVAM (2009). *ECVAM Skin Irritation Validation Study. Validation of the EpiSkin™ test method 15 min–42 hours for the prediction of acute skin irritation of chemicals*, 43pp. Ispra, Italy: European Commission, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods Available at: http://ecvam.jrc.it/ft_doc/EPISKIN-SIT-SOP% 20JC%20V13-23%2002%2009.pdf (Accessed 20.01.12).
30. Ale, I.S. & Maibach, H.I. (2008). Mechanisms in irritant and allergic contact dermatitis. In *Dermatotoxicology* (ed. H. Zhai, K-P. Wilhelm & H.I. Maiback), 7th edn, pp. 159–167. Boca Raton, FL, USA: CRC Press.
31. Coquette, A., Berna, N., Vandenbosch, A., Rosdy, M., De Wever, B. & Poumay, Y. (2003). Analysis of interleukin-1alpha (IL-1alpha) and interleukin-8 (IL-8) expression and release in *in vitro* reconstructed human epidermis for the prediction of *in vivo* skin irritation and/or sensitization. *Toxicology in Vitro* **17**, 311–321.

Genes and Environment, Vol. 34, No. 1 pp. 50–54 (2012)

Short communication

Tissue Sample Preparation for *In Vivo* Rodent Alkaline Comet Assay

Madoka Nakajima[1], Maya Ueda[1], Kohji Yamakage[2], Yuzuki Nakagawa[2], Munehiro Nakagawa[3], Wakako Ohyama[4], Takashi Omori[5], Norihide Asano[6], Makoto Hayashi[1] and Yoshifumi Uno[7,8]

[1]Biosafety Research Center, Foods, Drugs and Pesticides, Shizuoka, Japan
[2]Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan
[3]Drug Development Service Segment, Mitsubishi Chemical Medience Co., Ibaraki, Japan
[4]Yakult Central Institute for Microbiological Research, Tokyo, Japan
[5]Doshisha University, Kyoto, Japan
[6]Kinki University, Osaka, Japan
[7]Safety Research Laboratories, Mitsubishi Tanabe Pharma Co., Chiba, Japan

(Received May 12, 2011; Revised August 24, 2011; Accepted October 19, 2011)

The Japanese Environmental Mutagen Society/the Mammalian Mutagenicity Study group conducted a collaborative study to investigate whether cell nuclei or whole cells might be more suitably used to correctly detect genotoxic chemicals in the *in vivo* rodent alkaline Comet assay. Four participating laboratories applied four sample processing methods, i.e., three homogenization methods using the usual Potter-type shaft, a customized (loose) Potter-type shaft, or a Downs-loose-type shaft, for preparing cell nuclei, and the mesh membrane method for preparing whole cells, to the male rat liver. Homogenization with the usual Potter-type shaft clearly produced damage of the cell nuclei and DNA, while the other three methods seemed to provide similar conditions of the tissue samples. The proportion of cell nuclei: whole cells was 80–90%: 10–20% in all laboratories when the samples were prepared by homogenization using a Downs-loose-type shaft or by the mesh membrane method. The %DNA in tail were comparable in both samples among the negative control groups (single oral administration with physiological saline) of all laboratories, and showed an equal degree of increase in both samples of the ethyl methanesulfonate groups (single oral administration at 250 mg/kg) in all laboratories. In conclusion, the homogenization method using a loosely customized Potter-type shaft or a Downs-loose-type shaft, and the mesh membrane method would be equally acceptable for the *in vivo* rodent alkaline Comet assay.

Key words: *in vivo* Comet assay, tissue sample preparation, cell nuclei, whole cells

Introduction

Although the *in vivo* rodent alkaline Comet assay is widely used for detecting genotoxic chemicals, the standard protocol and the assay procedure, especially the method of preparation of free cells/nuclei, are still under debated. The International Workshop on Genotoxicity Testing (IWGT; 1,2) and the 4th International Comet Assay Workshop (3) proposed recommended protocols. Currently, the Japanese Center for the Validation of Alternative Methods (JaCVAM) has been coordinating an international validation study of the *in vivo* Comet assay to evaluate the assay capability for detecting genotoxic chemicals, and a standardized study protocol is already established based on the above recommendations and the pre-validation study results (4).

The largest data sets on the assay predictivity for chemical carcinogenicity have been established by Sasaki *et al.* (5,6), which are based on the analysis results of isolated cell nuclei prepared by a tissue homogenization method for many organs. The question has been raised about whether isolated cell nuclei or isolated whole cells may be more suitable to use in order to detect the genotoxic potential of test chemicals. The background for this question is the discrepant *in vivo* rodent Comet assay results published about *ortho*-phenyl phenol (OPP, CAS No. 90-43-7). Sasaki *et al.* reported that OPP showed positive results in the mouse using cell nuclei of the liver, kidney, lung and urinary bladder (7). On the other hand, Bomhard *et al.* obtained negative results in the same species (8). The only difference in the procedure used between Sasaki *et al.* and Bomhard *et al.* was in relation to the object isolated as

[8]Correspondence to: Yoshifumi Uno, Safety Research Laboratories, Mitsubishi Tanabe Pharma Co., 1-1-1 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan. Tel: +81-438-52-3562, Fax: +81-438-52-3542, E-mail: Uno.Yoshifumi@ma.mt-pharma.co.jp

© The Japanese Environmental Mutagen Society

the target for the Comet assay using the liver and kidney (8). One possible explanation for the difference in results was based on how the tissues were processed. Cell death is associated with increased levels of DNA strand breaks, and many researchers in this field feel that isolated cell nuclei might be damaged to a greater degree than isolated whole cells because of the homogenization process, resulting in increased false-positive results. Although there was much discussion on this subject in the 4th IWGT meeting, it was decided that more data were needed before a conclusion could be arrived at and that any international validation study should consider both processing methods (2).

Under this complex circumstance, the Japanese Environmental Mutagen Society/the Mammalian Mutagenicity Study group (JEMS/MMS) planned and coordinated a collaborative study to investigate which might be more suitable, cell nuclei or whole cells, for the *in vivo* rat alkaline Comet assay. Four laboratories took part in the collaborative study, applied both sample processing methods to the liver after oral administration to male rats of ethyl methanesulfonate at 250 mg/kg or physiological saline as the negative control, and then compared the assay data. Here, we report results of the collaborative study and discuss which might be more suitable, cell nuclei or whole cells, for the *in vivo* rat alkaline Comet assay.

Materials and Methods

Test chemicals: Ethyl methanesulfonate (EMS, CAS No. 62-50-0) was purchased from Sigma-Aldrich (St. Louis, MO, USA). EMS at 250 mg was dissolved in 10 mL of physiological saline immediately before administration. Physiological saline was also used as the negative control material.

Animals: Crj:CD(SD) male rats were obtained from Charles River Laboratories Japan (Kanagawa). The rats were housed in polycarbonate or stainless-steel cages under air-conditioned circumstances (20–24°C room temperature, 30–70% humidity, and 12 h-light/dark cycle), fed with rodent chow *ad libitum*, and given free access to tap water. The animals were quarantined and acclimated for at least 5 days, and then used at 8-week-old for the experiments.

Animal treatment: First, one non-treated rat was used for comparison of the isolation process of the cell nuclei and whole cells in each laboratory. Then, for the comparison between the isolated cell nuclei and the isolated whole cells, rats (three animals/group) were orally administered one dose of EMS at 250 mg/kg (10 mL/kg) or physiological saline at 10 mL/kg. Four laboratories participated in the former examination, and three in the latter.

Homogenizer shafts: Three types of homogenizer shafts were used for the isolation of the cell nuclei of the liver, i.e., a Potter-type shaft (Shaft A), a loosely customized Potter-type shaft (Shaft B), and a Downs-loose-type shaft (Shaft C). Shaft B was prepared by the following procedures: the Teflon part of Shaft A was ground with sand-paper and adjusted so as to not damage the cell nuclei and/or DNA (i.e., so as to allow loose contact with the homogenizer glass tube).

Isolation of cell nuclei and whole cells: Isolation of the cell nuclei with Shafts A and B was conducted by the method reported by Sasaki *et al.* (9), and that with Shaft C was done by the method reported by Miyamae *et al.* (10). The whole cells were isolated by the method reported by Tice (11). Animals were sacrificed by carbon dioxide inhalation or by cutting the abdominal aorta/vein under anesthesia (e.g., ethyl ether), and the liver was removed. A small piece of the liver (about 5 mm cube fragment) was minced with a pair of scissors and put into a glass tube with a chilled 75 mM sodium chloride solution containing 24 mM EDTA disodium, homogenized once (just one stroke, with the head of the shaft never reaching the bottom of the tube) with Shaft A or B at 600 rpm, and then centrifuged at 700 g for 10 min at 4°C to collect the cell nuclei. Accordingly, two samples of isolated cell nuclei were obtained as Sample A with Shaft A, and Sample B with Shaft B. Another piece of the liver was put into a glass tube with chilled phosphate-buffered saline [Ca^{2+} and Mg^{2+} free: PBS(−)] containing 54 mM EDTA, and then homogenized once (just one stroke) mildly by hand, and the suspension was passed through a nylon mesh (150 μm pore size, Tokyo Rikakikai, Tokyo), and a sample of isolated cell nuclei was obtained as Sample C. For the whole-cell isolation, the mesh membrane method was used. The liver fraction (about 5 mm cube fragment) was minced with a pair of scissors in chilled PBS(−) containing 54 mM EDTA, and the suspension was passed through a metal or nylon mesh (150 μm pore size), and Sample D was obtained as isolated whole cells.

Microscopic observation of the cell nuclei and whole cells: An aliquot of each sample was used for the Comet analysis described below. The remaining Samples C and D were centrifuged at 1500 rpm for 5 min in 4°C. The pellet was suspended and fixed with 5 mL of 10% neutral buffered formalin solution, and the suspension was mixed with Acridine orange (A.O.) solution at the ratio of 1:1. The mixture was immediately examined under a fluorescent microscope, and the ratio of cell nuclei and whole cells was calculated for 500 nuclei.

Comet analysis: The procedure has been described in previous reports (1–6). Briefly, an aliquot of each sample was embedded in 0.5% (w/v) low-melting agarose gel (Lonza, Rockland, ME, USA) on a slide. Two slides were prepared for each animal. The slides were immersed overnight in chilled lysing solution con-

taining 100 mM EDTA disodium, 2.5 M sodium chloride, 10 mM tris hydroxymethyl aminomethane, 1% (v/v) Triton-X100, and 10% (v/v) DMSO in purified water at pH 10, and then rinsed in purified water. The slides were covered with an alkaline solution consisting of 300 mM of sodium chloride and 1 mM of EDTA disodium in purified water at pH > 13 for 10 min, applied for electrophoresis at 1 V/cm and approximately 300 mA for 15 min below 4°C, and immersed for 10–20 min in neutralization buffer consisting of 0.4 M tris hydroxymethyl aminomethane in purified water at pH 7.5. Slides were dehydrated with absolute ethanol for at least 5 min, dried, and then stained with 20 μg/mL of ethidium bromide solution. Nuclei were scored with an image analysis system [Comet Assay IV (Perceptive Instrument Ltd., Suffolk, UK) or Rainbow Star System (Komet 4; Kinetic Imaging Ltd., Liverpool, UK)], and the %DNA in tail were calculated.

Results

In microscopic examination of the two homogenized samples prepared with Potter-type Shafts A and B, it seemed that both Samples A and B consisted of a lot of cell nuclei and few whole cells, and many more damaged cell nuclei and whole cells were observed in Sample A than in Sample B. In another homogenized sample C prepared with the Downs-loose-type Shaft C, there seemed to be a number of cell nuclei, but few whole cells. The sample condition of Sample C seemed similar to that of Sample B, i.e., containing lower numbers of damaged cell nuclei and whole cells. In Sample D, which was prepared with the mesh membrane method, the sample condition seemed to be the same as those of Samples B and C. In the Comet analysis performed with four samples, Sample A clearly showed DNA migration, even samples obtained from non-treated rats (Fig. 1a), and there was no obvious difference between Samples B, C and D prepared from the non-treated rats (Figs. 1b, 1c and 1d). Consequently, the % DNA in tail was increased in Sample A as compared with those in Samples B, C and D (Fig. 2). The number of cell nuclei and whole cells for Samples C and D was scored independently in three laboratories, and the ratio was determined to be 80–90% cell nuclei: 10–20% whole cells for both samples in all laboratories (Fig. 3). The Comet responses in the liver after *in vivo* treatment with EMS were also examined using Samples C and D. The %DNA in tail in both Samples C and D were comparable among the negative control groups in the three laboratories (Fig. 4). The average of these parameters increased similarly in both Samples C and D of the EMS group as compared with the finding in the negative con-

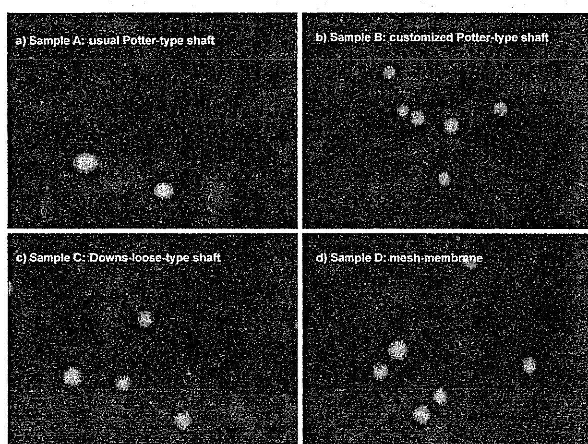

Fig. 1. Comet images obtained using the four sample preparation methods for the non-treated rat liver. Nuclei were stained with ethidium bromide solution. Sample A clearly showed DNA migration, and Samples B, C and D seemed to show no damage to the nuclei.

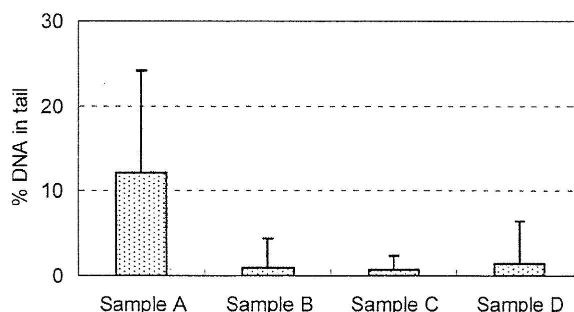

Fig. 2. %DNA in tail in Samples A, B, C and D. All samples were prepared from the liver of one non-treated rat, and analyzed with an image analysis system of Comet Assay IV. Each column shows the mean ± S.D. ($n = 100$ nuclei). The %DNA in tail of Sample A was higher than those of Samples B, C and D, indicating that the sample preparation procedure using the usual Potter-type shaft was associated with increased DNA migration.

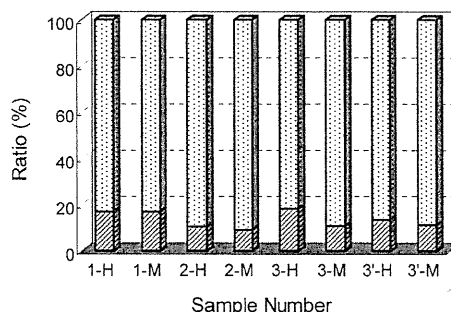

Fig. 3. Ratio of the cell nuclei (dot column) and whole cells (shadow column) in Samples C and D in three laboratories. The sample number shows the laboratory code: 1, 2 or 3, and H: homogenized sample obtained using a Downs-loose-type shaft, and M: samples obtained by the mesh membrane method. Laboratory 3 repeated the experiments, and the results are shown as 3′-H or 3′-M. The resultant ratio obtained was 80–90% cell nuclei: 10–20% whole cells for both samples in all laboratories.

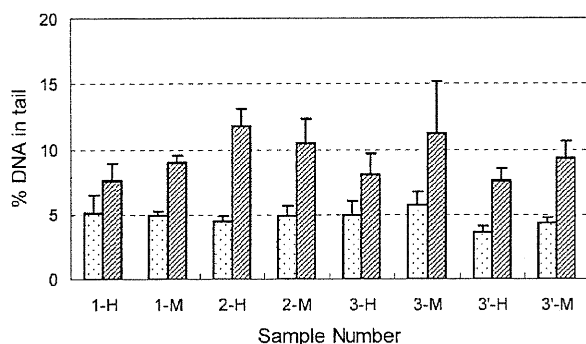

Fig. 4. %DNA in tail in Sample C prepared using a Downs-loose-type shaft and Sample D obtained using the mesh membrane method. Both samples were prepared from the liver of EMS-treated rats (shadow column) or saline-treated rats (dot column), and analyzed with an image analyzer of Rainbow Star System. Fifty nuclei/slide and two slides/animal were scored, and the mean of each animal was calculated. Each column shows the mean ± S.D. ($n=3$ animals). The sample number shows the laboratory code: 1, 2 or 3, and H: homogenized sample prepared using a Downs-loose-type shaft, or M: samples prepared using the mesh membrane method. Laboratory 3 repeated the experiments, and the results are shown as 3′-H or 3′-M. The average values of the parameters increased similarly in both Samples C and D of the EMS group as compared with those in the negative control groups in all three laboratories.

trol groups in the three laboratories, although the magnitude of responses in the EMS groups differed slightly among laboratories (Fig. 4).

Discussion

Three homogenizing procedures to prepare cell nuclei were used in the present study. In general, to prepare cell nuclei, a Downs-loose-type homogenizer shaft (Shaft C in this report) is suitable and useful for preventing damage of cell nuclei and/or DNA. However, Sasaki *et al.* who reported a huge database about *in vivo* rodent Comet assay (5,6) selected and used a Potter-type shaft and a homogenizer machine. Since the usual Potter-type homogenizer shaft (Shaft A in this report) is generally used to fragment tissue samples, Sasaki *et al.* slimmed the homogenizer shaft for use, described in this report as Shaft B (Sasaki, personal communication). In this study, Shaft A clearly produced damage of the cell nuclei and DNA. However, Shaft B yielded samples with a lower degree of damage of the cell nuclei and DNA (Figs. 1a, 1b and 2). The condition of the sample obtained using Shaft B seemed to be comparable to that obtained using Shaft C (Figs. 1b, 1c and 2). Therefore, it was considered that Shaft B (loosely customized Potter-type) and shaft C (Downs-loose-type) might be equally acceptable for preparation of the cell nuclei for the *in vivo* rodent Comet assay.

For the preparation of whole cells, the mesh membrane method was used in this study, which is the simplest and most popular method to prepare samples for the *in vivo* rodent Comet assay and is thus used in the JaCVAM initiative international validation study of the *in vivo* rodent Comet assay (4). However, surprisingly, the sample yielded by the mesh membrane method (Sample D in this report) contained numerous cell nuclei, but fewer whole cells, and the ratio of the cell nuclei and whole cells was similar to that in Sample C prepared using the Downs-loose type homogenizer (Fig. 3). In each of the three laboratories, the % DNA in tail in both Samples C and D were comparable in the negative control groups and the EMS-treated groups (Fig. 4). These data indicate that both sample preparation methods mainly provide cell nuclei and have no significant effect on the Comet assay results. The magnitude of the responses in the EMS groups seemed to be slightly different among laboratories, and these variations may raise some issues for our future validation study. The main purpose of this study, however, was not to obtain comparably positive results with EMS in all laboratories, but to examine whether comparable samples were obtained using different sample preparation methods, and thus these issues will be discussed after this study.

We could not obtain whole-cell samples using the mesh-membrane method. Another option to obtain intact whole cells is the enzyme digestion (e.g., collagenase-perfusion) method. Actually, some investigators have used this technique and the data on OPP by Bomhard *et al.* (8) were provided using an *in situ* perfusion technique for the liver and kidney. Therefore, the enzyme digestion method also needs to be examined in order to understand whether or not the discrepant results for OPP are truly related to the sample condition. However, the enzyme digestion methods require additional and complicated steps of cell preparation. When considering the actual status that the mesh membrane method has been the most widely and popularly used, additional investigation of the enzyme digestion method would seem to be less significant.

In conclusion, the homogenization method using a loosely customized Potter-type shaft or a Downs-loose-type shaft, and the mesh membrane method provided similar tissue sample conditions, with the samples mainly consisting of cell nuclei under the conditions used in this collaborative study. Thus, we consider that all of these methods might be equally acceptable for *in vivo* rodent alkaline Comet assay.

Acknowledgements: This study was supported by the JEMS/MMS.

References

1 Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu J-C, Sasaki YF. Single cell gel/comet assay: guidelines for *in vitro*

and *in vivo* genetic toxicology testing. Environ Mol Mutagen. 2000; 35: 206–21.

2 Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M, Sasaki YF, Thybaud V, Uno Y, Vasquez M, Hartmann A. Fourth International Workgroup on Genotoxicity Testing: Results of the *in vivo* Comet assay workgroup. Mutat Res. 2007; 627: 31–5.

3 Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. Recommendation for conducting the *in vivo* alkaline Comet assay. Mutagenesis. 2003; 18: 45–51.

4 JaCVAM protocol for international validation study. Available at: http://www.cometassay.com/index.htm (accessed 7 February 2012).

5 Sasaki YF, Sekihashi K, Izumiyama F, Nishidate E, Saga A, Ishida K, Tsuda S. The Comet assay with multiple mouse organs: comparison of Comet assay results and carcinogenicity with 208 chemicals selected from the IARC Monographs and U.S. NTP Carcinogenicity Database. Crit Rev Toxicol. 2000; 30: 629–799.

6 Sekihashi K, Yamamoto A, Matsumura Y, Ueno S, Watanabe-Akanuma M, Kassie E, Knasmüller S, Tsuda S, Sasaki YF. Comparative investigation of multiple organs of mice and rats in the Comet assay. Mutat Res. 2002; 517: 53–75.

7 Sasaki YF, Saga A, Akasaka M, Yoshida K, Nishidate E, Su YQ, Matsusaka N, Tsuda S. *In vivo* genotoxicity of ortho-phenylphenol, bisphenyl, and thiabendazole detected in multiple mouse organs by the alkaline single cell gel electrophoresis assay. Mutat Res. 1997; 395: 189–98.

8 Bomhard EM, Brendler-Schwaab SY, Freyberger A, Herbold BA, Leser KH, Richter M. O-phenylphenol and its sodium and potassium salts: a toxicological assessment. Crit Rev Toxicol. 2002; 32: 551–626.

9 Sasaki YF, Tsuda S, Izumiyama F, Nishidate E. Detection of chemically induced DNA lesions in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow) using the alkaline single cell gel electrophoresis (comet) assay. Mutat Res. 1997; 388: 34–44.

10 Miyamae Y, Yamamoto M, Sasaki YF, Kobayashi H, Igarashi-Soga M, Shimoi K, Hayashi M. Evaluation of a tissue homogenization technique that isolates nuclei for the *in vivo* single cell gel electrophoresis (comet) assay: a collaborative study by five laboratories. Mutat Res. 1998; 418: 131–40.

11 Tice RR. The single cell gel/comet assay: a microgel electrophoretic technique for the detection of DNA damage and repair in individual cells. In: Phillips DH, Venitt S, editors. Environmental mutagenesis. Oxford: Bios Scientific Publishers; 1995. p. 315–39.

Genes and Environment, Vol. 34, No. 1 pp. 18–24 (2012)

Regular article

Evaluation of the Genotoxicity of Aristolochic Acid in the Kidney and Liver of F344 *gpt* delta Transgenic Rat Using a 28-Day Repeated-dose Protocol: A Collaborative Study of the *gpt* delta Transgenic Rat Mutation Assay

Yuji Kawamura[1,6], Hiroyuki Hayashi[2], Osamu Tajima[3], Sayuri Yamada[3], Tomomi Takayanagi[4], Hisako Hori[4], Wataru Fujii[4], Kenichi Masumura[5] and Takehiko Nohmi[5]

[1]Toxicology Laboratory, Pharmaceutical Research Center, Meiji Seika Pharma Co., Ltd., Yokohama, Japan
[2]Research Planning & Management, R&D Planning & Management Dept., Meiji Seika Pharma Co., Ltd., Tokyo, Japan
[3]Food Safety Assurance Center, Quality Assurance & Environment Management Department, Kirin Group Office Co., Ltd.,Yokohama, Japan
[4]Safety Science Institute, Quality Assurance Division, Suntory Business Expert Ltd., Osaka, Japan
[5]Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan

(Received May 9, 2011; Revised June 20, 2011; Accepted July 5, 2011)

Transgenic rat gene-mutation assays can be used to assess genotoxicity of chemicals in target organs for carcinogenicity. Since gene mutations in transgenes are genetically neutral and thus accumulate along with treatment periods, the assays are suitable for genotoxicity risk assessment of chemicals using repeated-dose treatment methodologies. However, few studies have been conducted to examine the suitability of the assays in repeat-dose treatment protocols. In order to prove the utility of the transgenic rat assays, we treated *gpt* delta rats with aristolochic acid at 0.3 and 1 mg/kg by gavage daily for 28 days, and autopsied the rats 3 days after the final treatment, which is a protocol recommended by the International Workshop on Genotoxicity Testing (IWGT). Aristolochic acid exists in herbs and some other plants, and is carcinogenic in the kidney, bladder and stomach in rats. The mutant frequency (MF) in both the kidney and the liver increased significantly in a dose-dependent manner when the rats were treated with aristolochic acid. We concluded that the *gpt* delta rat assay is sensitive enough to detect gene mutations induced by aristolochic acid and also that the 28-day repeated-dose protocol is suitable for assessing genotoxicity of chemicals.

Key words: F344 *gpt* delta transgenic rat, aristolochic acid, 28-day repeated-dose protocol, *gpt* assay

Introduction

Transgenic gene-mutation assays are of a high value for the assessment of *in vivo* genotoxicity (1,2). In this method, mutations in reporter genes integrated in the rodent chromosomes can be identified in any organs/tissues after the reporter genes are recovered from the rodent cells to bacterial cells. Transgenic gene-mutation assays are suitable for the risk assessment of potential genotoxic chemicals dosed via repeated-dose treatment, since mutations can be analyzed in various time points during treatment and sampling periods (3). In addition, mutations in the reporter genes accumulate over time as the treatments progress (4,5). It is, therefore, expected that these assays enableus to assess the genotoxicity of chemicals with various dose levels, dosing periods and target organs.

Present issues to be solved for the use of transgenic gene-mutation assays include how the detection sensitivity can be confirmed and how the dosing periods can be standardized. In a genotoxicity assessment of 90 carcinogens, transgenic gene-mutation models are shown to have a high sensitivity and a good positive predictability (4). However, the majority of the 90 carcinogens assessed in that study are such strong mutagens that they could be used as positive controls in genotoxicity studies, and there are not enough data available on genotoxicants with a lower potency that allow assess-

[6]Correspondence to: Yuji Kawamura, Toxicology Laboratory, Pharmaceutical Research Center, Meiji Seika Pharma Co., Ltd., 760 Morooka-cho, Kohoku-ku, Yokohama 222-8567, Japan. Tel: +81-45-545-3175, Fax: +81-45-545-3152, E-mail: yuuji.kawamura@meiji.com

© The Japanese Environmental Mutagen Society