1. Introduction

A great emphasis is being placed more and more on non-animal skin sensitization tests due to animal welfare issues, cost, and technological limitations of in vivo assays. More recently, the 7th amendment of the EU requires non-animal testing to be developed for skin sensitization. Many researchers have tried several approaches in developing an in vitro skin sensitization test. One approach is to develop cellular-based methods (Ryan et al., 2001). Dendritic cells (DCs) including Langerhans cells (LC) play a key role in the initial process of skin sensitization. Therefore, DCs, including LCs, have been used for the development of in vitro skin sensitization tests. In vitro methods using human epidermal LCs based on the alteration of phenotype (HLA-DR and E-cadeherin) and function of LCs have been developed by Moulon et al. (1993), Krasteva et al. (1996), Verrier et al. (1999), and Rizova et al. (1999). But it is difficult to obtain a sufficient number of LCs from the epidermis, so this approach was limited. To solve this limitation, some researchers used human peripheral blood mononuclear cells (PBMC) or CD34+ hematopoietic progenitor cells (HPC) cultured in the presence of the specific cytokines as antigen-presenting cells instead of LCs. Some allergens such as DNCB, but not irritants such as SDS, caused an alteration of CD54, CD86 and HLA-DR surface expression (Aiba et al., 1997; Coutant et al., 1999). Other researchers also illustrated similar results that showed an increased expression of HLA-DR and CD86, and a decreased level of E-cadherin with the strong allergen Bandrowski's base but not with the irritant SDS (Rougier et al., 2000). De Smedt et al. (2002) illustrated that CD86 expression was the most reliable phenotypic marker for identification of allergens, but IL-1ß production was not concluded as an activation endpoint due to substantial individual variations. Changes in gene expression have also been evaluated as another approach for developing a sensitization alternative (Ryan et al., 2004). Although the use of DCs for predicting allergens has a potential, there are still some technical problems with the routine use of these cells in skin sensitization tests. These problems include availability of human blood and donor to donor variability (Aiba et al., 1997; Rougier et al., 2000). However, the use of human cell lines instead of DCs provided a technical solution to these latter problems. Iwamoto et al. (1999) established the human LC type DC cell line, ELD-1, and reported that this cell line has expression of CD1a, E-cadherin and MHC class II like LCs. The other cell line, KG-1 (human myeloid leukemia cell line), cultured in the presence of the specific cytokines was able to induce a potent allogenic T-cell response and upregulate CD86 expression following treatment with methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), but not with SDS (Hulette et al., 2001, 2002). A method using U-937 cells found that CD86 is good marker for distinguishing between allergens and irritants (Rousset et al., 2002; Ashikaga et al., 2003). Recently, we have reported an upregulation of CD86 and MHC class II internalization following exposure to some allergens such as DNCB and *p*PD when THP-1 cells were used (Ashikaga et al., 2002). Also augmentation of CD54 and CD86 expression on THP-1 cells treated allergens have been reported (Yoshida et al., 2003). In an earlier collaboration between our two laboratories (Kao and Shiseido), we optimized the protocol for an in vitro skin sensitization test using THP-1 and U-937 cells (refer to the related paper entitled "optimization of the h-CLAT protocol" within this journal). Once optimized, an inter-laboratory validation study using nine chemicals (six allergens and three non-allergens) was conducted between our two laboratories.

2. Materials and methods

2.1. Collaborating laboratories

Laboratory A, Kao Safety and Microbial Control Research Center; Laboratory B, Shiseido Safety and Analytical Research Center.

2.2. Cells and medium

THP-1 cells and U-937 cells from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 (Invitrogen Corp., Carlsbad, CA USA) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 0.05 mM 2-mercaptoethanol and 1% of antibiotic–antimycotic (Invitrogen Corp., Carlsbad, CA, USA).

2.3. Chemicals

All tested chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Allergens employed in the experiment were: dinitrochlorobenzene (DNCB), *p*-phenylenediamine (*p*PD), 2-mercaptobenzothiazole (2-MBT), nickel sulfate hexahydrate (Ni), cobalt sulfate heptahydrate (Co) and ammonium tetrachloroplatinate (Pt). The non-allergens were sodium lauryl sulfate (SLS), polyoxyethylene sorbitan monooleate (Tween 80) and dimethyl sulfoxide (DMSO). *p*PD, Ni, Co, Pt, SLS and Tween 80 were first solubilized in saline, while DNCB and 2-MBT were solubilized in DMSO. The final concentration of DMSO in culture media was always less than 0.2%. Globlins Cohn fraction II, III was obtained from SIGMA-Aldrich as an FcR blocking agent.

2.4. Chemical treatment of THP-1 and U-937

First, the MTT assay (Mossmann, 1983) was used to establish IC_{50} (μg/mL) values for each of the nine chemicals and each treatment period (24 h and 48 h). Cells

were plated at 1×10^6 cells/mL and treated using four concentrations of each chemical at 0.1×, 0.5×, 1×, and 2× IC_{50} for 24 h or 48 h. Once treated, FcR blocking procedure was conducted: 0.01% of Globlins Cohn fraction II, III were added for 10 min on ice. Then cells were stained with antibodies. At least two independent experiments were done for each concentration and time period.

2.5. Flow cytometric analysis and mAbs

After treatment with chemicals for 24 h or 48 h, the fluorescence intensities of the THP-1 cell and U-937 cell surface markers were analyzed by flow cytometry (Laboratory A: FACSCalibur CellQuest, Becton Dickinson, San Jose, CA, USA; Laboratory B: EPICS XL-MCL System II, Beckman Coulter Co., Ltd. Fullerton, CA, USA). Cell staining was performed using the following FITC-conjugated monoclonal antibodies (mAbs): anti-human CD54 (clone; 6.5B5) from DAKO (Glostrup, Denmark), anti-human CD86 (clone; Fun-1) from BD-PharMingen (San Diego, CA, USA); FITC labeled-mouse IgG1 (clone; DAK-G01) from DAKO. Using the manufacturer's recommended dilutions, cells were incubated with the above mAbs at 6 μL/3 × 10^5 cells/50 μL for the anti-human CD86 mAb, and 3 μL/3 × 10^5 cells/50 μL for the anti-human CD54 mAb. Also FITC labeled-mouse IgG1 as an isotype control was used at a dilution of 3 μL/3 × 10^5 cells/50 μL. These were treated for 30 min at 4 °C. After washing and resuspending with PBS supplemented containing 0.1% BSA, flow cytometry analysis was performed using each flow cytometry. Propidium iodide solution was used at a concentration of 0.625 μg/mL. After which, dead cells were gated out. A total of 10,000 living cells were analyzed. When the cell viability was less than 50%, relative fluorescence intensity (RFI) was not calculated because of diffuse labeling cytoplasmic structures due to cell membrane destruction (Becker et al., 1994). RFI was used as an indicator of CD86 and CD54 expression and was calculated by the following formula:

$$\mathrm{RFI}(\%) = \frac{\text{MFI of chemical-treated cells} - \text{MFI of chemical-treated isotype control cells}}{\text{MFI of vehicle control cells} - \text{MFI of vehicle isotype control cells}} \times 100$$

MFI = (Geometric) mean fluorescence intensity.

Two independent experiments were done for four doses (0.1×, 0.5×, 1×, and 2× IC_{50}) at each test period at two laboratories. Mean RFI values and cell viability (SD was not calculated) values were calculated from these two independent experiments. The IC_{50} value of each chemical is reported in Table 1. Cell viability was determined by PI analysis. If cell viability for one independent experiment was less than 50%, the data set for that concentration was not included in the evaluation.

Table 1
The IC_{50} (μg/mL) of test chemicals

Chemical	THP-1		U-937	
	24 h	48 h	24 h	48 h
DNCB	5	3	4	2
*p*PD	90	30	150	60
2-MBT	170	100	120	50
Ni	170	70	70	30
Co	130	70	50	30
Pt	100	50	40	40
SLS	90	70	90	70
Tween 80	1800	1600	1600	1100
DMSO[*1]	2500	2500	2500	2500

THP-1 and U-937 were evaluated to establish IC_{50} (μg/mL) values for each chemical using MTT-assay. Two separate exposure periods were evaluated for each cell line: 24 h and 48 h.

[*1] DMSO did not cause any cytotoxicity at 5000 μg/mL, which is the maximum concentration of this test. Therefore, the dose of 2× IC_{50} for DMSO was set 5000 μg/mL and IC_{50} of DMSO was 2500 μg/mL. Both cell lines were treated with four doses: 0.1×, 0.5×, 1× and 2× IC_{50}.

3. Results

3.1. Phenotypic characterization of THP-1 cells with 24 h treatment

Calculated RFI values for CD86 and CD54 expression following a 24 h treatment are shown in Fig. 1. The two laboratories had almost similar results in assessing changes in CD86 and CD54 expression. For CD86 expression, allergens except for 2-MBT augmented significantly RFI values compared to vehicle control and showed an RFI value over 150 at 0.5× or 1× IC_{50} (Pt tested in Laboratory B showed over 150 only at 0.1×). An RFI value of 200 or more was observed with DNCB, *p*PD, Ni and Co (only Laboratory B). For all non-allergens, the RFIs of CD86 were under 150, but Tween 80 of Laboratory A and DMSO of both laboratories were over 120. Three arbitrary thresholds were selected: RFI values of 120, 150, and 200. The accuracy of each threshold to correctly identify allergen from non-allergen was then evaluated. On the basis of these three criterions, the accuracy of Laboratory A was 67%, 89% and 67% for the criterion of 120, 150 and 200, respectively (Table 2). Laboratory B had a similar accuracy of 78%, 89% and 78%, respectively.

For CD54 expression, allergens had RFI values over 150 and 200. The exception was *p*PD which had an RFI value under 150 in Laboratory A and under 200 in both laboratories. 2-MBT did not up-regulate the expression of CD86, but the RFI value for CD54 expression was over 200 in

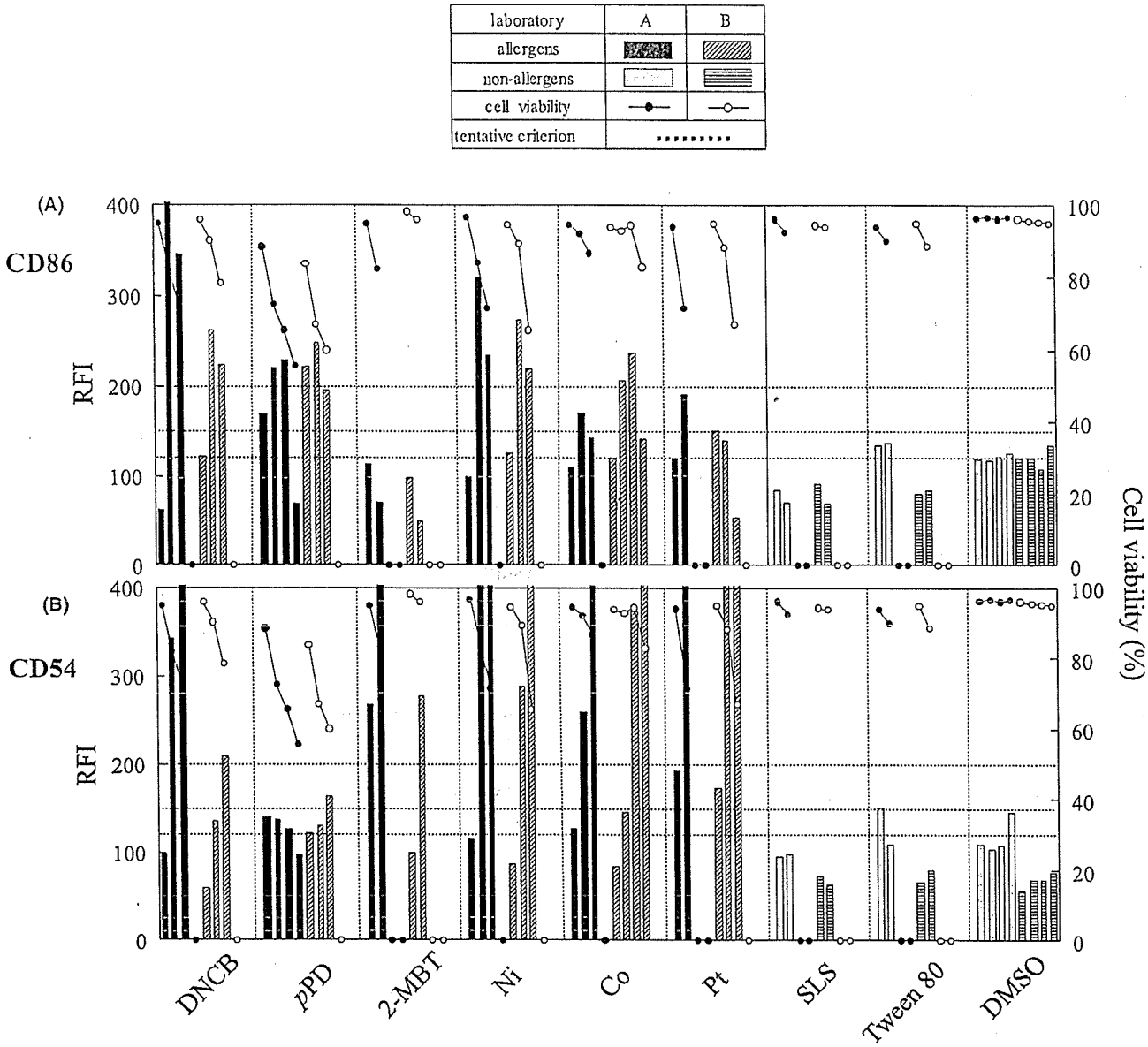

Fig. 1. The effect of chemical allergens and non-allergens on THP-1 cell expression of CD86 (A) and CD54 (B) for both laboratories at 24 h treatment. The cells were treated with nine chemicals at four concentrations. After the treatment, the expression of CD86 (A) and CD54 (B) was measured by flow cytometry and RFIs were calculated. The lines across the graph indicate RFI values of 120, 150 and 200.

both laboratories. The metal allergens, Ni, Co and Pt, had very high (over 500) RFI values. For non-allergens, the RFI values for Tween 80 and DMSO from Laboratory A were over 120 (Tween 80 was over 150). The other RFI values for the non-allergens were below 120. For the three criteria, the accuracy of Laboratory A was 78%, 78% and 89%, respectively, for CD54 expression and the accuracy of Laboratory B was 100%, 100% and 89%, respectively. Laboratory B had a little higher accuracy than Laboratory A.

From the above, expression of CD86 and CD54 gave good predictive results. The accuracy for CD86 and/or CD54 was calculated and an accuracy of 78/89%, 89/100%, and 100/100% was obtained for the lower, mid, and high criterion, respectively (Laboratory A/B data). The use of both markers improved the accuracy as compared with a single marker. Thus, the overall accuracy in the two laboratories was 78%, 89% and 100%, for our three tentative criterions, respectively (see Table 2).

3.2. Phenotypic characterization of THP-1 cells with 48 h treatment

Calculated RFI values for CD86 and CD54 expression following a 48 h treatment are shown in Fig. 2 and the summary data including accuracy are shown in Table 2. The two laboratories had almost similar results. Four doses for each chemical were tested but only one or two doses were evaluated due to the cytotoxicity of some chemicals (i.e., DNCB, *p*PD and Ni). The number of successful test

Table 2
The summary data for CD86 and CD54 expression following exposure to nine chemicals

	cell line	THP-1						U-937					
	treatment hours	24h			48h			24h			48h		
	tentative criterion	120	150	200	120	150	200	120	150	200	120	150	200
CD86	DNCB	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/+	-/+	-/+	-/+	-/+
	p PD	+/+	+/+	+/+	+/+	+/+	+/-	+/+	+/+	-/+	+/+	+/+	-/+
	2-MBT	-/-	-/-	-/-	-/-	-/-	-/-	-/+	-/+	-/-	-/+	-/+	-/+
	Ni	+/+	+/+	+/+	+/+	+/-	-/-	+/+	+/-	-/-	-/+	-/+	-/-
	Co	+/+	+/+	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-	-/-	-/-
	Pt	+/+	+/+	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
	SLS	-/-	-/-	-/-	-/-	-/-	-/-	-/+	-/-	-/-	-/+	-/-	-/-
	tween 80	+/-	-/-	-/-	-/-	-/-	-/-	+/+	-/-	-/-	-/-	-/-	-/-
	DMSO	+/+	-/-	-/-	-/+	-/-	-/-	-/+	-/-	-/-	-/+	-/-	-/-
	accuracy (K/S)	67 / 78	89 / 89	67 / 78	89 / 78	89 / 78	78 / 67	67 / 67	78 / 89	56 / 78	67 / 67	56 / 89	44 / 78
CD54	DNCB	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-
	p PD	+/+	-/+	-/-	+/+	-/+	-/-	+/+	-/-	-/-	-/+	-/-	-/-
	2-MBT	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-
	Ni	+/+	+/+	+/+	+/-	+/-	-/-	-/+	-/-	-/-	-/-	-/-	-/-
	Co	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/-	-/-	-/-	-/-
	Pt	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/-	-/-	-/+	-/-	-/-
	SLS	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	tween 80	+/-	+/-	-/-	+/+	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	DMSO	+/-	-/-	-/-	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	accuracy (A/B)	78 / 100	78 / 100	89 / 89	89 / 67	89 / 78	78 / 78	44 / 78	33 / 33	33 / 33	33 / 56	33 / 33	33 / 33
combined accuracy *1		*78 / 89*	*89 / 100*	*100 /100*	*89 / 78*	*100 / 78*	*89 / 78*	*67 / 67*	*78 / 89*	*56 / 78*	*67/ 67*	*56 /89*	*44 / 78*
inter-laboratory accuracy *2		*78*	*89*	*100*	*78*	*78*	*78*	*44*	*67*	*56*	*33*	*56*	*44*

Relative fluorescence intensities (RFIs) of CD86 and CD54 expression for THP-1 and U-937 were compared with three tentative criteria. The accuracy was calculated based on these results. Each column shows the data of Laboratory A on the left and the data of Laboratory B on the right (Laboratory A data/ Laboratory B data). "+" means RFI of CD86 and/or CD54 ⩾ tentative criterion, and "−" means RFI of CD86 and CD54 < tentative criterion. "−/−" on allergens means that both laboratories obtained false negative results, and "+/+" on non-allergens meant that both laboratories obtained false positive results. "+/−" and "−/+" on allergens means one of laboratories had false negative results. "+/−" and "−/+" on non-allergens means one of laboratories had false positive results. *1 = the accuracy of CD86 and/or CD54. If either RFI of CD86 or CD54 was more than criterion, the chemical would be "+", positive. And if both RFI of CD86 and CD54 were below criterion, the chemical would be "−", negative. *2 = the accuracy that both laboratories could predict exactly based on either CD86 and/or CD54.

doses at 48 h treatment was fewer than the 24 h treatment. But both CD86 and CD54 expression tended to be similar at 24 h and 48 h.

For CD86 expression, allergens except for 2-MBT for both labs and Ni in Laboratory B augmented significantly the RFI values compared to vehicle control and the RFI values were over 150. Concerning the RFI values for non-allergens, only DMSO in Laboratory B was over 120, but below 150. The RFI values for the other non-allergens were all below 120 and did not differ compared to vehicle control. The accuracy was 89/78%, 89/78% and 78/67% for the tentative criterion of 120, 150 and 200, respectively (Laboratory A/B data).

For CD54 expression, allergens had RFI values over 150 and 200. The exceptions were *p*PD, which had an RFI value under 150 in Laboratory A and under 200 in both laboratories, and Ni in Laboratory B, which had an RFI value under 150 and under 200 in both laboratories. 2-MBT did not up-regulate the expression of CD86, but the RFI values of CD54 expression in both laboratories were over 200. Ni did up-regulate significantly in the 24 h treatment but did not change much especially for Laboratory B. For metal allergens, Co and Pt had very high RFI values (over 500). For non-allergens, RFI values for Tween 80 in Laboratory B were over 150, but below 200 (RFI = 189), and RFI values for DMSO were over 120 only for Laboratory B. The RFI values for the other non-allergens were below 120. The accuracy was 89/67%, 89/78% and 78/78% for the tentative criterion of 120, 150 and 200, respectively (Laboratory A/B data).

From the above, expression of CD86 and CD54 on THP-1 cells were also good predictive markers following a 48 h treatment. The accuracy for CD86 and/or CD54 expression was calculated and an accuracy of 89/78%, 100/78% and 89/78% was obtained for the tentative criterion of 120, 150 and 200, respectively (laboratory A/B data). The use of both markers improved the accuracy as compared with a single marker. The overall accuracy for

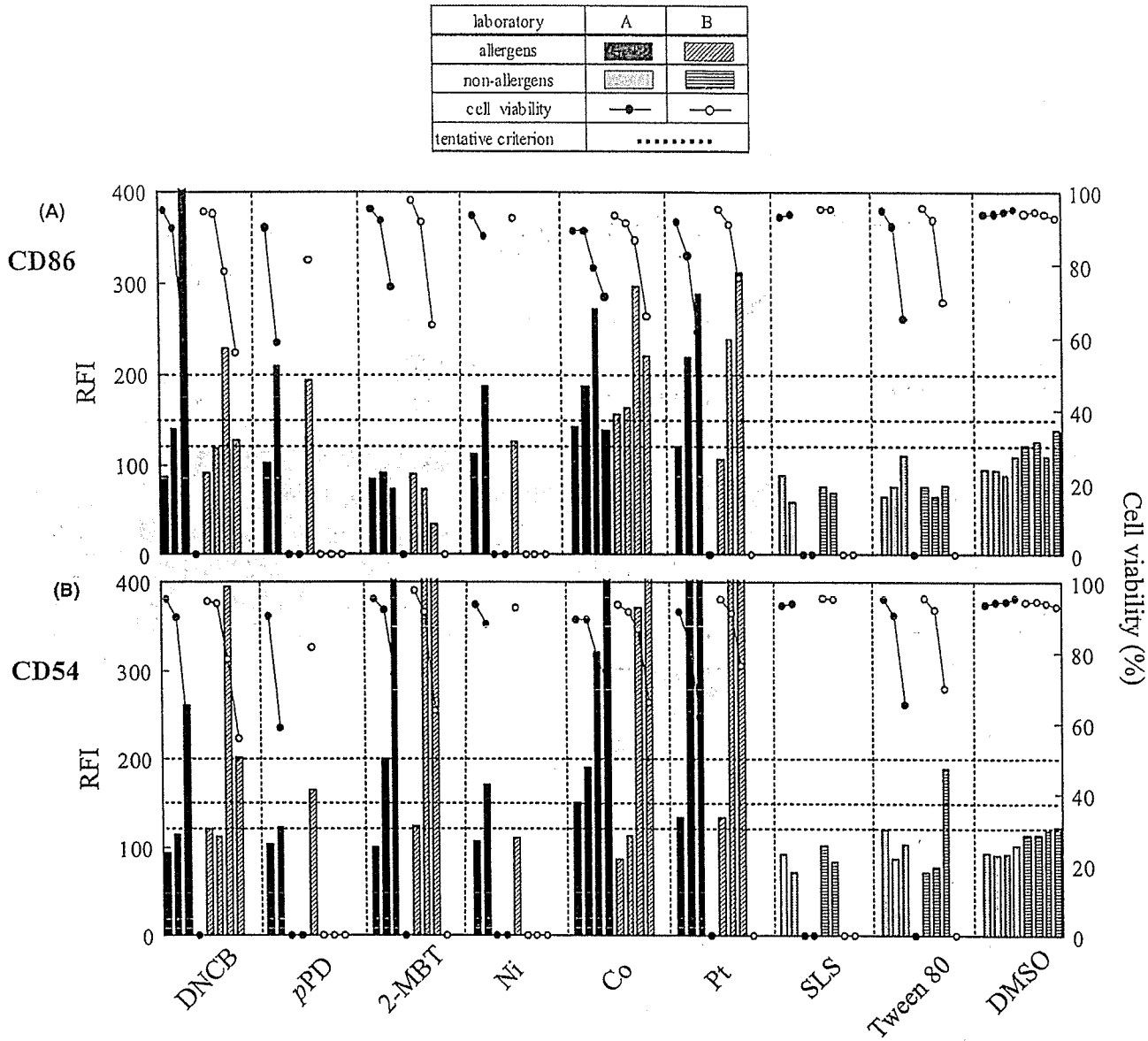

Fig. 2. The effect of chemical allergens and non-allergens on THP-1 cell expression of CD86 (A) and CD54 (B) for both laboratories at 48 h treatment.

the two laboratories was 78% for our three tentative criteria (120, 150, and 200, respectively; see Table 2). These accuracies were not better than those noted in the 24 h treatment experiments.

3.3. Phenotypic characterization of U-937 cells with 24 h treatment

The data are shown in Fig. 3. The reproducibility of cell viability for the two laboratories was not better than with THP-1 cells for the same concentrations. For DNCB, Laboratory A could evaluate only one dose ($0.1\times IC_{50}$) but Laboratory B could evaluate three doses ($0.1\times$, $0.5\times$ and $1\times IC_{50}$).

For CD86 expression, the RFI values for *p*PD, Co, and Pt were over 150 at both laboratories, but DNCB, 2-MBT and Ni were up-regulated over 150 for only one laboratory. For the non-allergens, all RFI values were under 150, but Tween 80 in Laboratory A and three non-allergens in Laboratory B were over 120. The accuracy was 67/67%, 78/89% and 57/78% for the lower, mid, and high criterion, respectively (Laboratory A/B data).

No difference was noted in CD54 expression between cells treated with most allergens compared to cells treated with vehicle control. Some allergens up-regulated CD54 expression over an RFI value of 120 at Laboratory A and/or B, but did not reach an RFI value over 150. The RFI values for all non-allergens were below 120. The accuracy was 44/78%, 33/33% and 33/33% for the three criteria, respectively (Laboratory A/B data).

The accuracy for CD86 and/or CD54 expression was calculated and an accuracy of 67/67%, 78/89% and 56/78%, respectively, was obtained for the three criteria (Laboratory A/B data). When comparing the ability of either marker to correctly identify each test material at

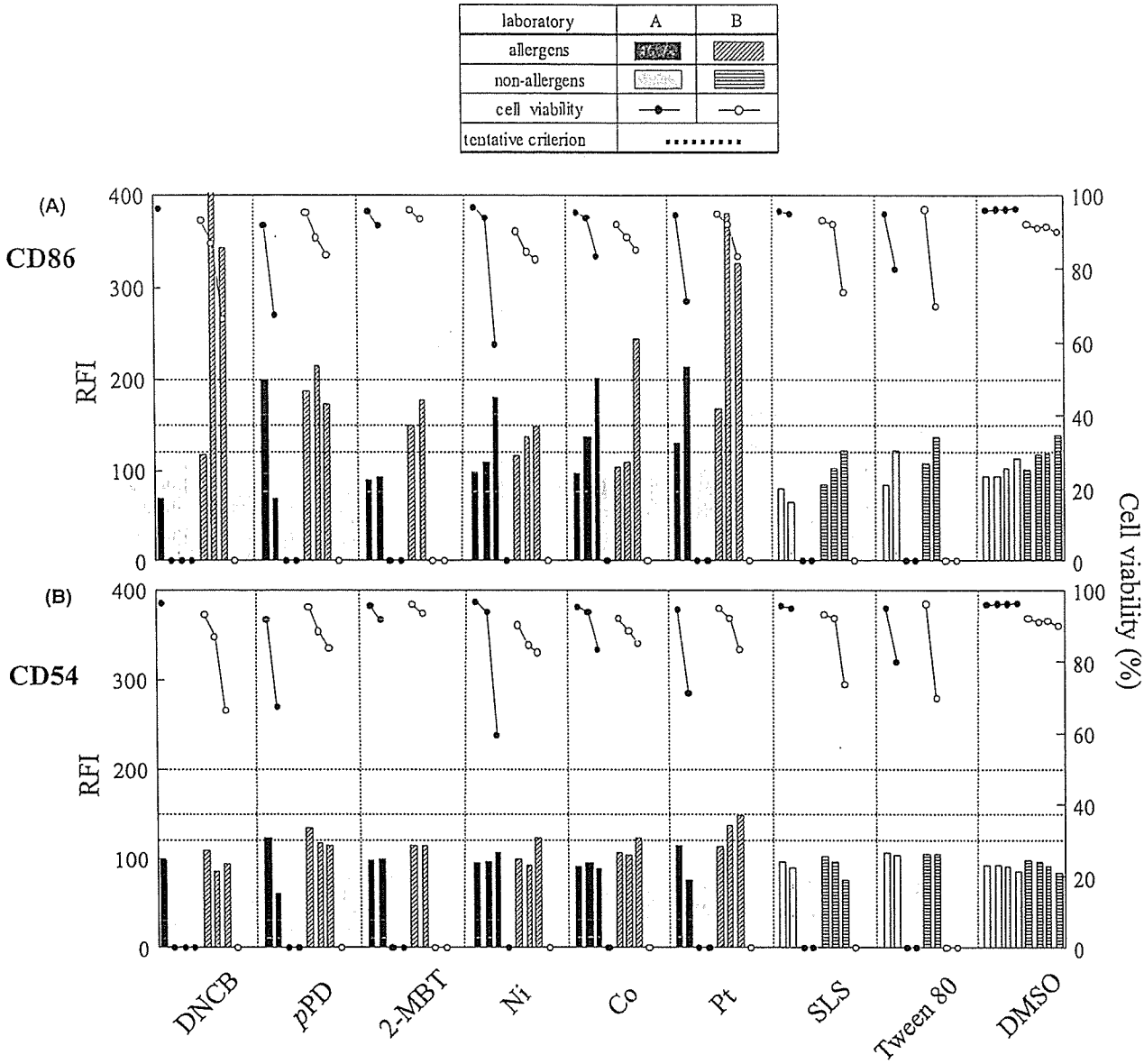

Fig. 3. The effect of chemical allergens and non-allergens on U-937 cell expression of CD86 (A) and CD54 (B) for both laboratories at 24 h treatment.

each threshold selected, the accuracy of the CD86 and/or CD54 marker was similar to only using the CD86 marker; so no improvement was obtained as compared with a single marker. The overall accuracy for the two laboratories was 44%, 67% and 56%, respectively, for our three tentative criteria (120, 150, and 200, respectively; see Table 2) and these accuracies were not better than those observed with THP-1 cells at 24 h treatment.

3.4. Phenotypic characterization of U-937 cells with 48 h treatment

The data are shown in Fig. 4. RFI values for CD86 and CD54 expression tended to be similar to the results obtained with U-937 cells at 24 h treatment. The accuracy for CD86 expression was 67/67%, 56/89% and 44/78%, respectively, and 33/56%, 33/33% and 33/33% for CD54 expression, respectively, for the criterion of 120, 150 and 200 (Laboratory A/B data). The accuracy for CD86 and/or CD54 expression was 67/67%, 56/89% and 44/78%, respectively (Laboratory A/B data). As with the THP-1 cell experiments, the use of both markers did not improve the overall accuracy compared to only using a single marker. So, no improvement was noted as compared with a single marker. The overall accuracy of the two laboratories was 33%, 56% and 44% for our three tentative criteria, respectively (120, 150 and 200, see Table 2). These accuracies were not better than those observed with THP-1cells treated for 24 h and 48 h or with U-937 cells treated for 24 h.

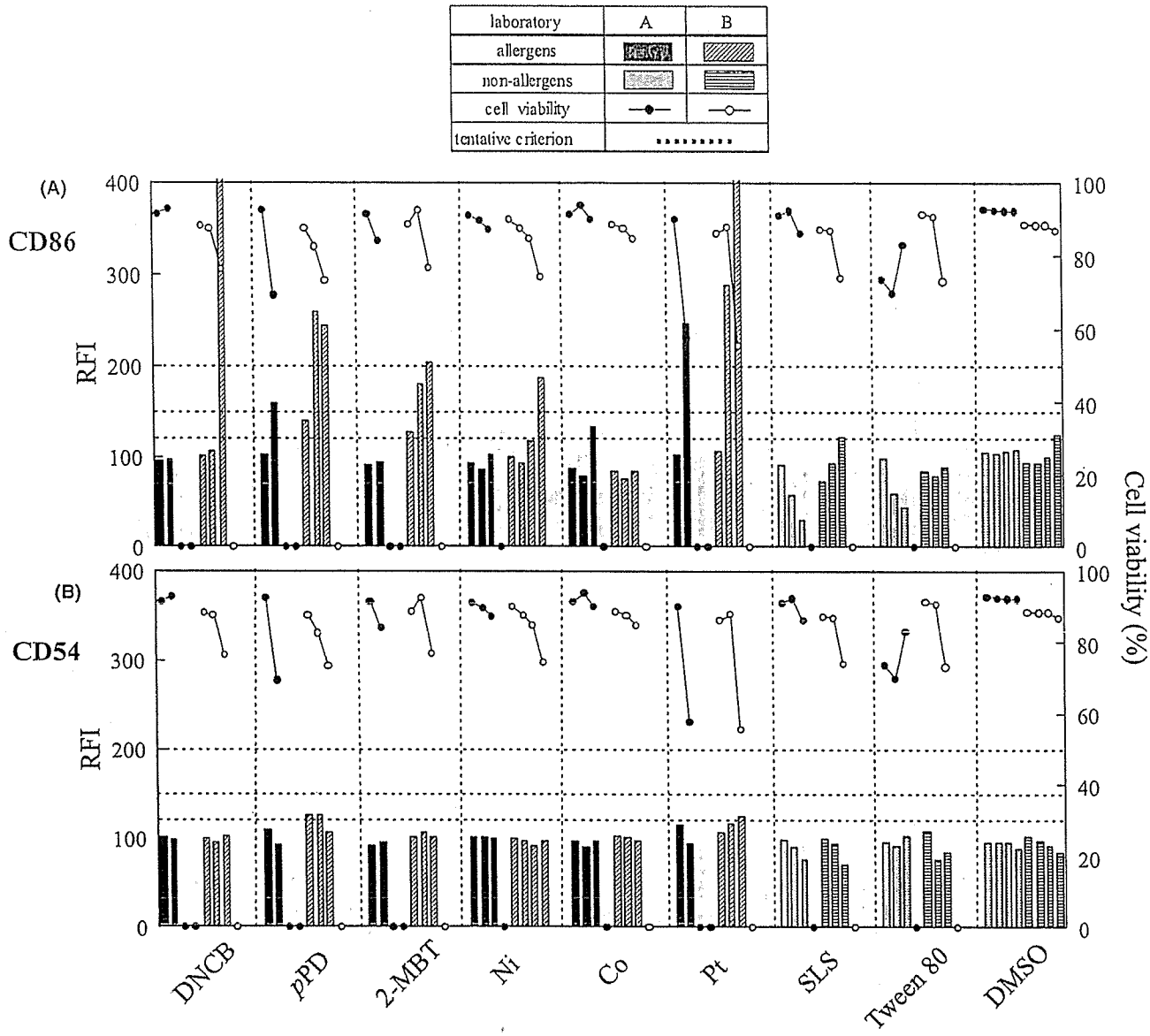

Fig. 4. The effect of chemical allergens and non-allergens on U-937 cell expression of CD86 (A) and CD54 (B) for both laboratories at 48 h treatment.

3.5. Relationship between up-regulation of CD86/CD54 expression and cell viability

The cell viability is shown in Table 3 for cells treated with concentrations of chemical that enhanced RFI values over 150 or 200. Depending on allergen, appropriate cell viability for up-regulation of CD86/CD54 expression was not the same. For CD86 expression, each allergen had an RFI value over 150 with a cell viability ranging from 60% to 97% (Laboratory A) or 60% to 97% (Laboratory B). RFI values over 200 were observed at a cell viability range from 62% to 93% (Laboratory A) and 60% to 93% (Laboratory B). The pattern of CD86 expression observed primarily for each set of treatments had one RFI maximum value as concentration increased. The cell viability range from 55% to 97% (55–95%) for Laboratory A and 60% to 95% (63–95%) for Laboratory B were needed for up-regulation of CD54 expression (over 200 RFI). In this case, observed CD54 expression resulted in a classical dose response pattern. With increasing concentration, the RFI also increased for most chemicals. For U-937 cells, some level of cytotoxicity was also needed for up-regulation of CD86/CD54 expression (data not shown).

4. Discussion

Our two laboratories have independently explored the use of human cell lines in an in vitro skin sensitization test. Ashikaga et al. (2002, 2003) indicated that up-regulation of CD86 expression and MHC class II molecule internalization on THP-1 and U-937 cells occurred when these cells were treated with some allergens. They suggested that the

Table 3
The cell viability that showed over 150 or 200 RFI values in THP-1 cells at 24 h treatment are shown

	CD86	CD54
(A) Lab. A		
DNCB	68, 76, 76, 88	68, 76, 76, 88
*p*PD	62, 69, 71, 75, 84, 93	84
2-MBT	–	87, 78, 95
Ni	60, 68, 75, 84, 84	60, 68, 75, 84, 84
Co	88, 85, 93, 91	64, 88, 85, 93, 91
Pt	80, 97	55, 64, 80, 92, 97
(B) Lab. B		
DNCB	76, 81, 89, 92, 96	76, 81, 89
*p*PD	60, 60, 63, 71, 77, 91	60, 60, 63, 77
2-MBT	–	96, 95
Ni	78, 53, 90, 89, 97	78, 53, 90, 89
Co	89, 97, 92, 93, 93	89, 78, 97, 92, 93
Pt	94	71, 63, 87, 89, 94, 95

The cell viability was from the independent data of six allergens. The upper table (A) was for Laboratory A and the lower table (B) was for Laboratory B. Data on the left were for the RFI values of CD86 and data on the right were for the RFI values of CD54. The bold character represents the cell viability at the dose which the RFI value was over 200. A certain level of cytotoxicity (from 55% to 97% cell viability) was needed for enhancement of CD86/CD54. Values presented in table are percent (%).

test system using this phenotypical alteration can be used as an in vitro sensitization test. In addition, Yoshida et al. (2003) and Sakaguchi et al. (2003) showed that not only CD86 expression but also CD54 expression on THP-1 and KG-1 cells could be used in the development of an in vitro method. To develop an in vitro skin sensitization test that could be accepted by regulatory authorities, both laboratories collaborated to optimize a protocol in order to begin validation of the method (refer to the related paper entitled "Optimization of the h-CLAT Protocol" within this journal). Using the optimized protocol, we evaluated a total of nine chemicals (six allergens, three non-allergens) for their potential to augment expression of CD86 and CD54 markers in this inter-laboratory validation study. When a criterion is defined for CD86 and/or CD54 expression, allergens/non-allergens were better predicted using THP-1 cells compared to U-937 cells following a 24 h or 48 h treatment time. Especially if our tentative criterion is defined as an RFI of 200, the accuracy of the method was 100% for both laboratories with THP-1 cells treated for 24 h. On the other hand, the accuracy in two laboratories with U-937 cells was only 56%. One of the reasons for the low prediction rate with U-937 cells was the inherent high expression levels of CD54 compared to THP-1 cells. In our untreated U-937 cells, we observed a high expression level of CD54 (Fig. 5). Champagne et al. (1998) also showed a high expression of CD54 on untreated U-937 cells. Due to the high expression of CD54, significant increases in CD54 expression could not be obtained in U-937 cells when treated with allergens. In our study, when looking only at CD86 expression, the accuracy of the two laboratories at 24 h treatment was respectively 89%/89% (THP-1) and 78%/89% (U-937) for the criterion of RFI = 150. So, no big differences were noted between THP-1 and U-937.

We used CD86 and CD54 expression as markers for sensitization in the validation study. Expression of CD86 and CD54 were good predictive markers when THP-1 cells were used, but for U-937 cells treated at 24 h and 48 h,

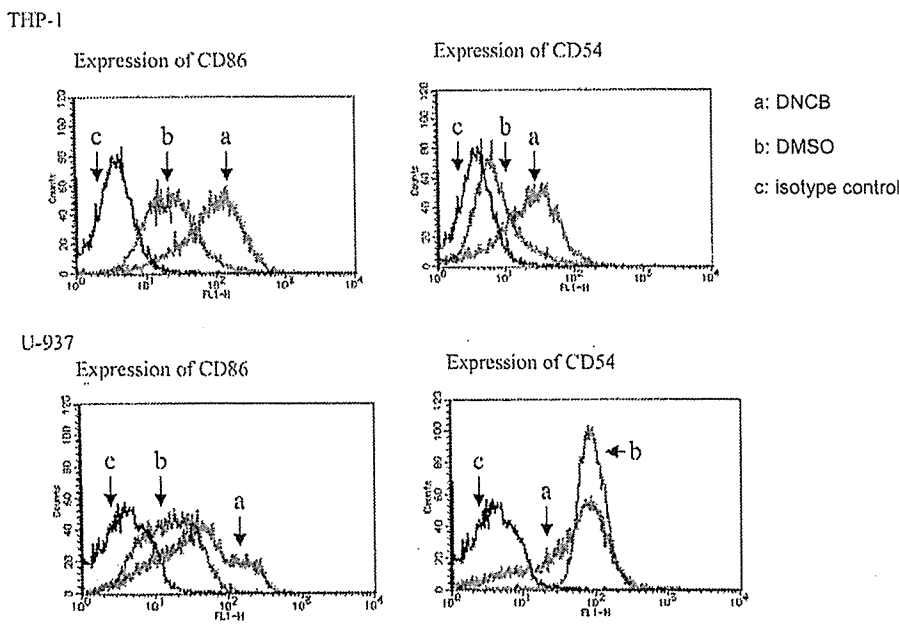

Fig. 5. Histogram of flow cytometric analysis for CD86 and CD54 on THP-1 and U-937 following DNCB treatment. The upper histograms were for THP-1 and the lower histograms were for U-937. The left histograms were for CD86 and the right histograms were for CD54. These histograms were for Laboratory A using FACSCalibur. Each figure had three lines: (a) was for DNCB, (b) was for DMSO and (c) was for Isotype control.

expression of CD86 was a better predictor than CD54 expression. For THP-1 cells, the accuracy also improved when both markers (CD86 and CD54) were used as compared with a single marker. There are some human cell line studies using CD86 expression as a marker. Rousset et al. (2002) evaluated CD86 expression for over 50 chemicals using U-937 cells and reported an increase of CD86 expression with treatment of allergens. Hulette et al. (2002) used cytokine-induced KG-1 cells (a human myeloid cell line) to evaluate two allergens (i.e., dinitorofluorobenzene and MCI/MI), and an irritant (i.e., SDS) following a 48 h treatment. Only MCI/MI increased expression of CD86 to 194% of untreated control. Python et al. (2004) demonstrated that the exposure to allergens (TNBS, 2-MBT) induced an increase of CD86 expression, whereas irritants (SDS, methyl salicylate) showed no relevant modulation of CD86 on U-937 cells treated with IL-4.

When we compared the 24 h and 48 h treatment times, the 24 h treatment time tended to have a better accuracy than the 48 h treatment time for THP-1 cells. For U-937 cells, there was not a significant difference between these two treatment times. Rousset et al. (2002) evaluated the kinetics of CD86 expression on U-937 cells using flow cytometry following $NiSO_4$ treatment and demonstrated that a 48 h treatment was better than a 24 h treatment. Python et al. (2004) also evaluated kinetics of CD86 expression on U-937 cells following TNBS treated with IL-4 and showed CD86 expression increased with time after 24 h, 48 h, and 72 h at the same tested concentrations compared to control. In our test protocol, test concentrations and treatment times were established following a cytotoxicity assay using MTT. This process differs from previous studies, which may account for the observed differences between our results and those from other investigators.

In this study, we set three tentative criteria: RFI values of 120, 150, and 200. For THP-1 cells exposed for 24 h, RFI values $\geqslant 200$ would be the best criterion for the nine chemicals tested. But the criterion of RFI value $\geqslant 50$ also has a similar prediction potential. Previously, we reported that an RFI value of 120 or greater in THP-1 cells can be used as a criterion for judging whether a test substance is positive or not (Ashikaga et al., 2002; Yoshida et al., 2003). The observed higher response (RFI value $\geqslant 200$) may be from the optimization of the test protocol with THP-1 cells. For U-937 cells, Rousset et al. (2002) set a value of 120 as an induction index ((assay/control) $\times$ 100) and showed that the concordance with human clinic data was over 90% for over 50 chemicals when this index was used as a criterion for allergens/non-allergens. From these data including our results, the criterion for predicting allergens with an in vitro skin sensitization test using human cell lines was a in the range of 120–200. To decide the final value, more data is needed.

As we show in Table 3, up-regulation of CD86 and CD54 expression was observed with concentrations that result in a cell viability range from about 60% to 95%. From these data, low levels of cytotoxicity may be an integral part in this method for predicting an allergen. In our previous study, Yoshida et al. (2003) reported that many allergens showed a positive response at 70–85% cell viability and that a subtoxic concentration would be a key factor in detecting an allergen. Ashikaga et al. (2002) also showed that a subtoxic allergen concentration resulted in an up-regulation of CD86 expression. Similarily, Rousset et al. (2002) also reported increased expression of markers on U-937 cells with a >70% cell viability with their test method. In summary, the use of a subtoxic allergen concentration appears to be a key factor in predicting an allergen when using human cell lines in an in vitro skin sensitization method. To develop a more robust method, we need to further evaluate the role a subtoxic concentration plays in the prediction of an allergen in this assay.

Recently the development of in vitro safety testing including skin sensitization methods is becoming more arduous due to methodology limitations, animal welfare considerations, and regulatory perspectives. Several investigators have reported using human cells, peripheral blood-derived dendritic cells, or have evaluated changes in expression of cell surface markers (e.g., CD54, CD80, CD83, CD86, and chemokine receptors: CXCR4, CCR5 and CCR7) in the development of their in vitro alternative tests (Rustemeyer et al., 2003; Aeby et al., 2004; Staquet et al., 2004; Boisleve et al., 2004). On the other hand, methods using human cell lines are not as common. In this study, we conclude that our method, human cell line activation test (h-CLAT) using THP-1 and U-937 cells, but especially THP-1 cells with a 24 h treatment, was a robust and reliable method. This method may be a useful in vitro skin sensitization model to predict various contact allergens. To establish the h-CLAT, we need to actively advance a multi-laboratory validation study and optimize the protocol further in near future.

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Existence of a no effect level for MeIQx hepatocarcinogenicity on a background of thioacetamide-induced liver damage in rats

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As exposure to heterocyclic amines might increase the risk of liver cancer, we investigated the carcinogenic potential of MeIQx under conditions of liver damage caused by TAA. Male, 6-week-old F344 rats ($n = 280$) were divided into 14 groups; groups 1–7 received TAA (0.03% in drinking water) and groups 8–14 received water for the first 12 weeks. Thereafter, the animals received MeIQx at doses from 0, 0.001, 0.01, 0.1, 1, 10 to 100 p.p.m. (groups 1–7 and 8–14, respectively) in pellet basal diet for 16 weeks. All survivors were killed at week 28 for assessment of numbers and areas of GST-P positive foci, considered to be pre-neoplastic lesions of the liver. Values were increased significantly in all the groups receiving TAA→MeIQx compared to MeIQx alone ($P < 0.01$). Numbers of GST-P positive foci were significantly increased in groups 7 and 14 (treated with 100 p.p.m. MeIQx) as compared to 0 p.p.m.-MeIQx (groups 1 and 8) ($P < 0.01$), along with areas in group 14 compared to group 8 ($P < 0.01$). However, with the maximum likelihood method, the data for numbers of GST-P positive foci (groups 1–7 and groups 8–14) fitted the hockey stick regression model, representing no differences from groups 1–5 and from groups 8–13, despite a linear dose-dependent increase of MeIQx-DNA adducts from 0.1 to 100 p.p.m. We conclude that there is a no effect level for MeIQx hepatocarcinogenicity, even on a background of TAA-induced liver damage. (*Cancer Sci* 2006; 97: 453–458)

Exposure to dietary and environmental carcinogens in our everyday lives may be closely related to the occurrence of cancer.[1,2] Of many genotoxic carcinogens occurring naturally in our environment, interest has been concentrated on heterocyclic amines generated during the frying or grilling of foods, especially meat and fish products.[3]

It has been generally considered that genotoxic carcinogens have no threshold with regard to their carcinogenic potential. However, this concept is theoretical and has been founded on the limited data available for cancer risk assessment of genotoxic carcinogen exposure.[4,5] Therefore it is important to estimate the incidence and severity of adverse effects in human populations at actual exposure levels, which means that the focus should be on relatively low doses.[6–8]

MeIQx is a genotoxic heterocyclic amine causing DNA adducts[9,10] and tumors with long-term treatment in mice and rats.[11–13] It is likely that exposure to MeIQx may be particularly harmful in persons suffering from liver disease, such as cirrhosis, and this needs to be taken into account in risk assessment and management. In the present study, we experimentally induced liver damage in rats using TAA, a well-known hepatotoxin, before feeding MeIQx at doses from low to high levels, and evaluated quantitative data for GST-P positive foci, which are considered to be pre-neoplastic lesions of the liver,[14–16] MeIQx DNA adducts, and 8-OHdG in the liver. Because recent research has indicated that dietary factors can affect DNA methylation,[17,18] an examination of this parameter was included.

Materials and Methods

Animals and treatment

Male, 6-week-old F344 rats ($n = 280$) were obtained from Charles River Japan (Atsugi, Japan) and housed in a room with a 12 h light/dark cycle, at constant temperature and humidity. Rats were allowed free access to pellet chow diets (MF-1, Oriental Yeast, Tokyo, Japan) during the experiment and all procedures were approved by the Institutional Animal Care and Use Committee.

Animals were divided into 14 groups: groups 1–7 received TAA (Sigma-Aldrich, St Louis, MO, USA) in drinking water at a concentration of 0.03% for 12 weeks, while groups 8–14 were given drinking water without TAA. Thereafter, they received MeIQx (purity 99.9%, Nard Institute, Nishinomiya, Japan) at doses of 0 (groups 1 and 8), 0.001 (groups 2 and 9), 0.01 (groups 3 and 10), 0.1 (groups 4 and 11), 1 (groups 5 and 12), 10 (groups 6 and 13), or 100 p.p.m. (groups 7 and 14) in pellet basal diet for 16 weeks continuously. All survivors were killed at week 28 under ether anesthesia.

Bodyweight, food consumption and water intake of all animals were measured every week. At necropsy, liver weight was measured, and liver tissue was fixed in 10% phosphate-buffered formalin and routinely processed for embedding in paraffin, and staining of 4-μm sections with hematoxylin and eosin and Azan-Mallory for histopathological examination. Further sections were applied for immunohistochemical

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Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; GST-P, glutathione S-transferase placental form; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; TAA, thioacetamide.

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staining. The remaining liver samples from all animals were snap-frozen in liquid nitrogen for subsequent biochemical analyses.

Immunohistochemical examination of GST-P

The avidin–biotin complex method was used to demonstrate GST-P in sections (4 μm) of liver tissue dewaxed with xylene and hydrated through a graded ethanol series. Sections were treated sequentially with 0.3% hydrogen peroxide, normal goat serum, rabbit anti-GST-P antibody (MBL, Nagoya, Japan) at 1:1000 dilution, biotin-labeled goat anti-rabbit IgG and avidin–biotin–peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA, USA). Immune complexes were visualized with 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. As a negative control, normal serum was used instead of primary antibodies. The sections were counterstained with Mayer's hematoxylin to facilitate examination under a light microscope.

Quantification of GST-P positive foci

GST-P positive foci (having more than two positive cells) of all animals were counted under a light microscope. Total areas of GST-P positive foci and of the entire liver sections were measured using a color image processor (IPAP, Sumica Technos, Osaka, Japan) to allow calculation of the number of foci per cm^2 and the area (mm^2) per cm^2 of liver section. Data were the mean ± SD value for all samples per group.

Quantification of MeIQx-DNA adduct levels in the livers

The levels of MeIQx-DNA adducts were measured by the ^{32}P-postlabeling method under modified adduct intensification conditions using frozen samples, as previously reported.[19] Data were the mean ± SD value from three samples per group and three independent experiments.

Quantification of 8-OHdG formation

DNA samples isolated from pieces of frozen liver weighing 500 mg were digested into deoxynucleosides by combined treatment with nuclease P1 and alkaline phosphatase. Levels of 8-OHdG were determined by high-performance liquid chromatography (HPLC), as described earlier,[20] and results were expressed as the number of 8-OHdG residues/10^5 total deoxyguanosines. Data were the mean ± SD value for 10 samples per group and four independent experiments.

Immunohistochemical staining of 5-methylcytosine

The avidin–biotin complex method was used to demonstrate 5-methylcystosine in sections (4 μm) of liver tissue dewaxed with xylene and hydrated through a graded ethanol series. Sections in sodium citrate buffer (pH 6.0) were boiled in an autoclave for 25 min and then treated with 3.5 N HCl and 3% hydrogen peroxide, normal horse serum, and anti-5-methylcytosine antibody (Calbiochem, La Jolla, CA, USA) at 1:1000 dilution, followed by ABC-peroxidase procedures (ABC kit). Immune complexes were visualized with 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. As a negative control, normal serum was used instead of primary antibodies. The sections were counterstained with Mayer's hematoxylin to facilitate examination under a light microscope.

Statistical analysis

The Tukey–Kramer method was applied to the data of GST-P positive foci, MeIQx-DNA adducts and 8-OHdG levels using the JMP program (SAS Institute, Cary, NC, USA). For all comparisons, P-values less than 5% ($P < 0.05$) were considered to be statistically significant. For analysis of the dose–response relationship for the data of GST-P positive foci, the data of numbers of GST-P were divided into two categories (groups 1–7 and groups 8–14), and the maximum likelihood method[21] was used to obtain optimally fitted models using the SAS/IML procedure (SAS Institute).

Results

Body and relative liver weights, and histopathological examination of liver

Marked growth retardation was noted during TAA treatment, with a significant decrease in final bodyweights compared with the corresponding non-TAA treatment groups (Table 1,

Table 1. Final bodyweights and relative liver and spleen weights

Group	TAA	MeIQx (p.p.m.)	Number of rats	Initial bodyweight (g)	Final bodyweight (g)	Relative organ weight (%)	
						Liver	Spleen
1	+	0	30	120.9 ± 5.4	331.1 ± 24.4**	3.2 ± 1.1*	0.2 ± 0.1*
2	+	0.001	30	118.8 ± 5.2	334.4 ± 23.3**	3.2 ± 1.1*	0.3 ± 0.1*
3	+	0.01	30	120.5 ± 5.6	331.5 ± 20.9**	3.4 ± 1.3**	0.3 ± 0.1**
4	+	0.1	30	121.9 ± 5.2	339.2 ± 21.0**	3.0 ± 0.8	0.2 ± 0.1
5	+	1	30	120.7 ± 5.5	337.6 ± 20.8**	2.9 ± 0.8	0.2 ± 0.1
6	+	10	30	120.2 ± 4.9	343.6 ± 19.7**	2.9 ± 0.6	0.2 ± 0.0
7	+	100	29	121.2 ± 5.0	326.0 ± 16.3**	3.3 ± 0.7	0.3 ± 0.1*
8	–	0	10	119.5 ± 6.8	390.8 ± 11.9	2.1 ± 0.1	0.2 ± 0.0
9	–	0.001	10	119.0 ± 6.0	404.8 ± 19.2	2.1 ± 0.1	0.2 ± 0.0
10	–	0.01	10	117.9 ± 4.9	403.9 ± 14.8	2.1 ± 0.1	0.2 ± 0.0
11	–	0.1	10	118.1 ± 5.5	399.5 ± 27.2	2.1 ± 0.2	0.2 ± 0.0
12	–	1	10	118.9 ± 3.1	399.5 ± 18.8	2.1 ± 0.1	0.2 ± 0.0
13	–	10	10	119.3 ± 5.9	394.5 ± 18.1	2.1 ± 0.1	0.2 ± 0.0
14	–	100	10	118.1 ± 6.2	389.9 ± 21.3	2.3 ± 0.1	0.2 ± 0.0

Data represent the mean ± SD. *,** Significantly different from the corresponding control group without thioacetamide (TAA) at $P < 0.05$ and $P < 0.01$, respectively. MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline.

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Table 2. Number and area of glutathione *S*-transferase placental (GST-P) positive foci

Group	TAA	MeIQx (p.p.m.)	Number of rats	Number (no./cm²)	Area (mm²/cm²)
1	+	0	30	21.836 ± 5.615*	6.291 ± 4.065*
2	+	0.001	30	21.074 ± 7.218	6.558 ± 3.651*
3	+	0.01	30	19.626 ± 6.597*	5.845 ± 3.856*
4	+	0.1	30	19.734 ± 6.708*	4.521 ± 3.246*
5	+	1	30	20.237 ± 6.115*	3.841 ± 1.261*
6	+	10	30	25.740 ± 7.942*	5.033 ± 2.771*
7	+	100	29	40.671 ± 12.187*,**	6.291 ± 2.529*
8	–	0	10	0.698 ± 0.844	0.002 ± 0.003
9	–	0.001	10	0.778 ± 0.746	0.001 ± 0.002
10	–	0.01	10	0.679 ± 0.441	0.001 ± 0.001
11	–	0.1	10	0.735 ± 0.894	0.002 ± 0.003
12	–	1	10	0.538 ± 0.475	0.002 ± 0.002
13	–	10	10	1.010 ± 0.981	0.001 ± 0.001
14	–	100	10	7.504 ± 1.977**	0.024 ± 0.012**

Data represent the mean ± SD. *Significantly different from the corresponding control group without thioacetamide (TAA) at $P < 0.01$. **Significantly different from control group (group 1 or 8) at $P < 0.01$. MeIOx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline.

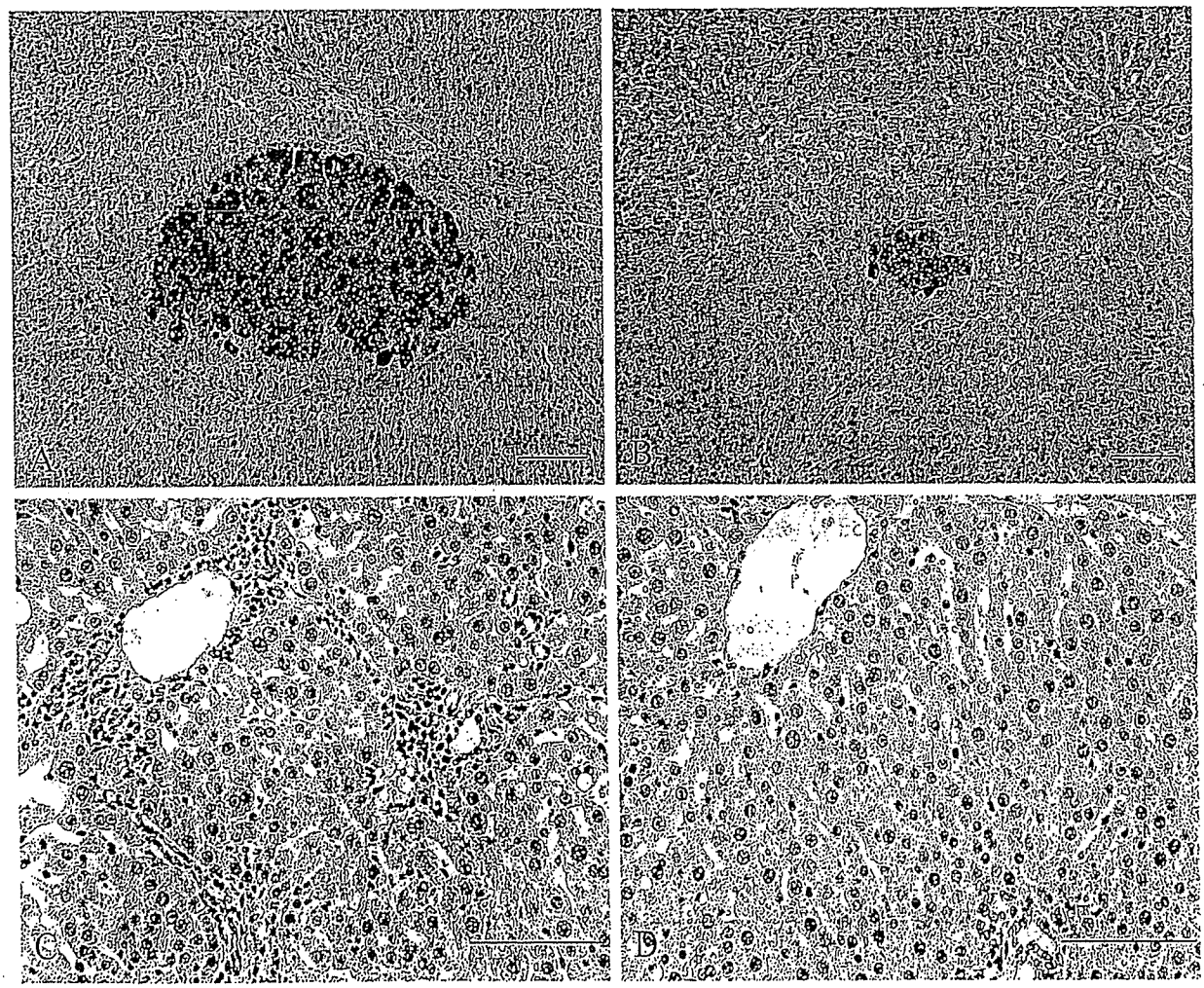

Fig. 1. Immunohistochemical staining of glutathione *S*-transferase placental form (GST-P) positive foci and 5-methylcytosine in liver of rats. (A) GST-P positive foci in a rat treated with 0.03% thioacetamide (TAA) in the drinking water for 12 weeks then 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) 100 p.p.m. for 16 weeks. (B) GST-P positive foci in a rat treated with MeIQx 100 p.p.m. for 16 weeks. (C) 5-methylcytosine in the liver from a rat treated with 0.03% TAA in the drinking water for 12 weeks then MeIQx 100 p.p.m. for 16 weeks; note many hepatic cells in hepatic nodules show negative staining, but non-parenchymal cells are positive. (D) 5-methylcytosine in the liver from a rat treated with normal diet (MeIQx 0 p.p.m.) for 16 weeks; most hepatic cells show positive staining; bar = 100 μm.

$P < 0.01$). There was a significant increase of relative liver and spleen weight in groups 1, 2 and 3 compared with that of groups 8, 9 and 10 ($P < 0.05$). Liver of animals treated with TAA showed cirrhosis and/or fibrosis, with nodular areas of hepatocytes, separated by fibrous septa with a collagenous matrix and myofibroblast-like cells and fibroblasts. There was one unscheduled death in group 7 during the experiment.

Quantitative data for GST-P positive foci

TAA treatment induced bigger GST-P positive foci compared to MeIQx alone (Table 2 and Fig. 1A,B). Numbers and area of GST-P positive foci increased significantly in all groups receiving TAA→MeIQx compared to MeIQx alone ($P < 0.01$). Moreover, the numbers of GST-P positive foci were significantly increased in groups 7 and 14 (treated with 100 p.p.m. MeIQx) as compared to 0 p.p.m.-MeIQx (groups 1 and 8) ($P < 0.01$), while the areas were significantly increased in group 14 compared to group 8 ($P < 0.01$). However, there were no differences at 10 p.p.m. or below as compared to control levels, with or without TAA.

With the maximum likelihood method, the data of numbers of GST-P positive foci (groups 1–7 and groups 8–14) fitted the hockey stick regression model, with no differences from groups 1–5 and from groups 8–13 (Fig. 2A and 2B, respectively).

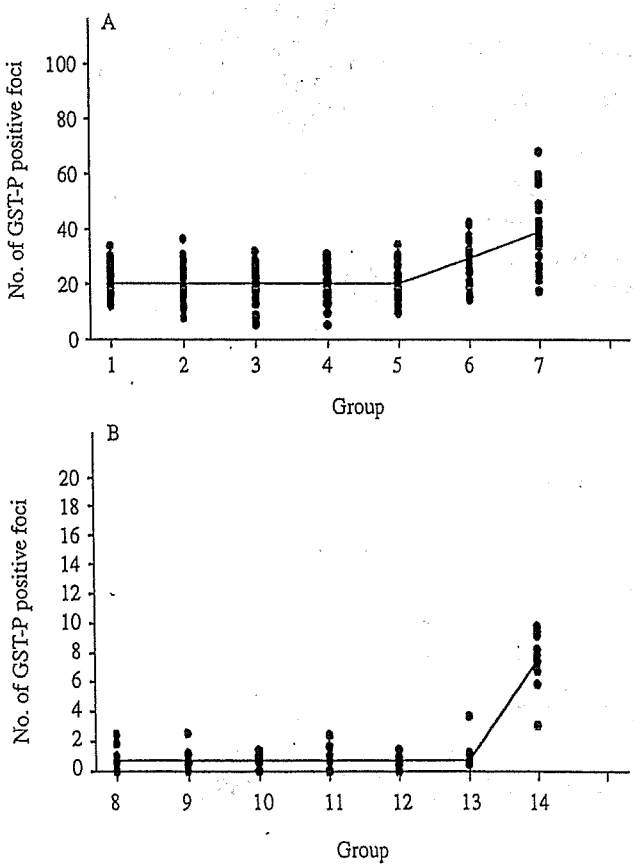

Fig. 2. Analysis of the dose–response relationship for the number of glutathione *S*-transferase placental form (GST-P) positive foci. Statistical analyses were carried out as detailed in the Materials and Methods section. (A) Numbers of GST-P positive foci demonstrated no differences from groups 1–5. (B) Numbers of GST-P positive foci demonstrated no significant variation from groups 8–13.

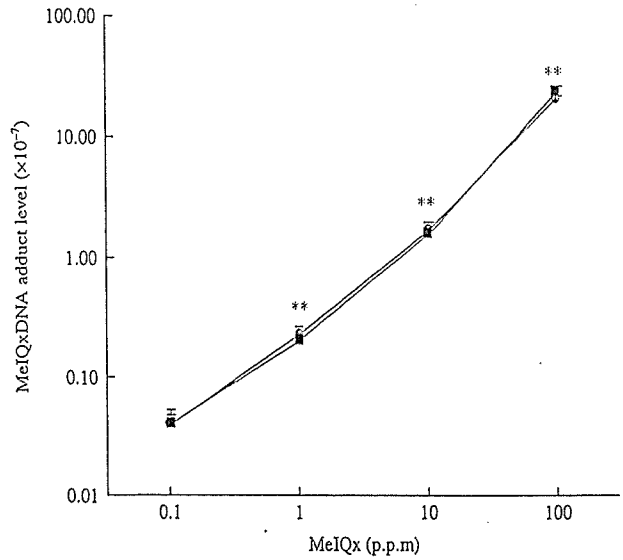

Fig. 3. 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)-DNA adduct levels in the livers of rats treated with MeIQx, with or without thioacetamide (TAA). Levels of MeIQx-DNA adducts in the liver were measured as detailed in the Materials and Methods section. Data are the mean ± SD values from three samples per group and three independent experiments. (O) TAA and MeIQx combined treatment groups; (□) MeIQx alone groups. **Significantly different from MeIQx 0.1 p.p.m. ($P < 0.01$).

MeIQx-DNA adduct levels in liver

Linear dose-dependent increase of MeIQx-DNA adducts was evident from 0.1 to 100 p.p.m. (Fig. 3). However, there were no differences between the groups treated with TAA→MeIQx and those exposed to MeIQx alone. With the low MeIQx intake of groups below 0.01 p.p.m., no adducts could be detected in either TAA→MeIQx or MeIQx alone treated animals; their levels might be under the detection limit ($<5 \times 10^{-10}$).

Quantitative data for 8-OHdG

HPLC analysis of 8-OHdG formation showed that there were no differences among the groups (Fig. 4), including the TAA→MeIQx and MeIQx alone groups.

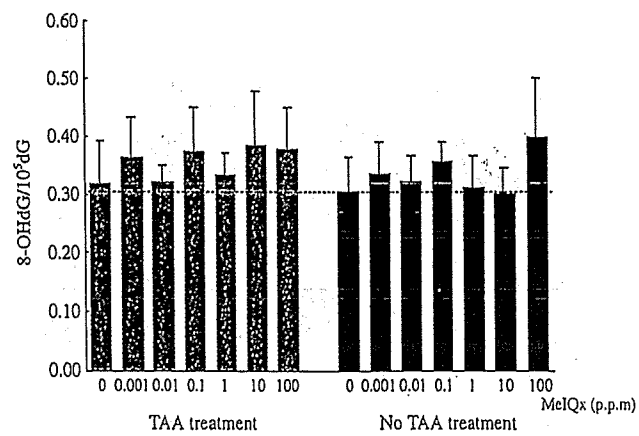

Fig. 4. 8-OHdG formation levels in the livers of rats treated with 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), with or without thioacetamide (TAA). Levels of 8-OHdG were determined as detailed in the Materials and Methods section, and expressed as numbers of residues/10^5 total deoxyguanosines. Data are the mean ± SD values from 10 samples per group and four independent experiments.

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Immunohistochemical staining of 5-methylcytosine in liver

Around nodules, non-parenchymal cells such as fibroblasts or myofibroblasts and bile ductular cells showed positive staining, but many cells in hepatic nodules were negative (Fig. 1C). However, most nuclei in livers from rats treated with normal diet (MeIQx 0 p.p.m.) showed positive staining (Fig. 1D).

Discussion

We have shown earlier that low-dose treatment with MeIQx results in elevation of DNA adducts, but no induction of GST-P positive foci, 8-OHdG and mutation frequency in rat liver.[6,7,22] These experimental results are line with the data for several genotoxic carcinogens such as *N*-nitrosodiethylamine, *N*-nitrosodimethylamine, 2-amino-1-methyl-6-phenolimidazo [4,5-*b*]pyridine and 2-acetylaminofluorene.[6–8,23–25] In the present study, the modifying effect of MeIQx on GST-P positive foci was more marked with numbers than areas, but in both cases there were no effect levels at lower doses regarding induction of GST-P positive foci, with or without TAA treatment.

The dose–response pattern analysis of the foci using the maximum likelihood method showed numbers to fit the hockey stick regression model, with no differences from groups 1–5 and from groups 8–13. Although shortening of no effects levels, it is particularly important that there were no effects even on a background of liver damage.

No effect levels with biological responses may fluctuate with health status and other exposures,[26] and may be associated with repair of DNA damage[27,28] and homeostatic mechanisms to maintain cellular equilibrium and normal function.[29] However, as there may be a particular individual susceptibility at a low dose,[30] intermittent exposure of genotoxic carcinogens may be related to greater risk of genotoxic damage.[31] Therefore, it is important to assay potential damage induced by genotoxic carcinogens under complex conditions in further studies.

Our previous findings on cotreatment of MeIQx with CCl_4 also showed no effect levels for the induction of GST-P positive foci in liver of rats with mild fibrosis.[32] Compared with the CCl_4 method, continuous administration of TAA in the drinking water is a non-invasive approach for chronic induction of fibrosis and/or cirrhosis in large numbers of animals.[33,34] In this study, we could evaluate the carcinogenic potential of MeIQx without any chemical interference, and found treatment of TAA induced liver fibrosis and/or cirrhosis without mortality.

It is considered that DNA adduct formation is a good marker for exposure to several carcinogens.[35,36] While an increase in MeIQx-DNA adducts was noted with dose-dependence, the additional liver disease did not exert any influence in the present study. Furthermore, formation of 8-OHdG showed no differences among the groups. Compared with previous reports,[7,37,38] it seems older animals show higher background levels of 8-OHdG and there is low sensitivity to MeIQx treatment.

Immunohistochemical staining of 5-methylcytosine provided evidence that hepatic nodules were characterized by hypomethylation status. Interestingly, around nodules, non-parenchymal cells such as fibroblasts or myofibroblasts and bile ductular cells showed positive staining. As involvement of global hypomethylation and regional hypermethylation of tumor suppressor genes in carcinogenesis has been proposed,[39,40] alteration of methylation in genotoxic carcinogen-induced tumors warrants further investigation.

In conclusion, in the present study, there was a no effect level regarding hepatocarcinogenicity of MeIQx, even on a background of TAA-induced liver damage.

Acknowledgments

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ORIGINAL ARTICLE

The confidence interval of allelic odds ratios under the Hardy–Weinberg disequilibrium

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Abstract In single nucleotide polymorphism (SNP) data analysis, the allelic odds ratio and its confidence interval (CI) are usually used to evaluate the association between disease and alleles at each SNP. The usual formula for calculating the CI of the allelic odds ratio based on the Hardy–Weinberg equilibrium (HWE) may, however, lead to errors beyond the control assured by the nominal confidence level if HWE is not true. We therefore present a generalized formula for CI that does not assume HWE. CIs calculated by this generalized formula are likely to be wider than those by the usual method if the Hardy–Weinberg disequilibrium (HWD) is toward a relative deficiency of the heterozygotes (fixation index greater than 0), whereas they are likely to be narrower if HWD is toward a relative excess of the heterozygotes (fixation index less than 0). A simulation experiment to examine the influence of the generalization was performed for the case where 2% of SNPs had a fixation index greater than 0. The result revealed that the generalized method slightly decreased the mean number of falsely detected SNPs.

Keywords Allelic odds ratio · Confidence interval · Fixation index · Hardy–Weinberg disequilibrium · SNP

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Introduction

Recently, we have witnessed the completion of the project for identifying the human genome sequence (International Human Genome Sequencing Consortium 2004), the accumulation of enormous SNP-related data into public databases (Sachidanadam et al. 2001; Haga et al. 2002), and the development of high throughput SNP typing technologies. This progress has provided modern molecular biology with an ability to identify a genotype (combination of alleles) at any particular genetic locus for a large number of individuals (Hirschhorn et al. 2005).

In genetic association studies, the phenotype of interest is typically associated with an allele or genotype for biallelic markers, such as SNPs, and consequently many researchers are interested in calculating the allelic odds ratio and its confidence interval (CI) for identifying SNPs that may have a close association, e.g., to a certain disease. The usual method, which calculates the CI using Eq. 1 based on the logarithm $\left(\log \hat{\psi}\right)$ of the estimated allelic odds ratio, the upper $\alpha/2$ quantile ($z_{\alpha/2}$) of the standard normal distribution, and observed frequencies n_{ij}'s in Table 1 (Balding et al. 2001), assumes the Hardy–Weinberg equilibrium (HWE) in study populations.

$$\exp\left(\log\hat{\psi} \pm z_{\alpha/2}\cdot\sqrt{\frac{1}{2n_{11}+n_{21}}+\frac{1}{2n_{31}+n_{21}}+\frac{1}{2n_{12}+n_{22}}+\frac{1}{2n_{32}+n_{22}}}\right). \quad (1)$$

Hardy–Weinberg disequilibrium (HWD) is often encountered when experimental errors occur in the SNP typing. However, even after the careful quality control of the genotyping, the genotype distribution may depart from HWE for a variety of other reasons, such as stratification, selection, inbreeding, assortative or disassortative mating (Wright 1951, 1965; Nei 1987). Under such a Hardy–Weinberg disequilibrium (HWD), the standard error of the estimated allelic odds ratio given in the last term of Eq. (1) will either be overestimated or underestimated. In order to solve this problem, Schaid and Jacobsen (1999) provided a correction method based on determining the correct variance for the observed allele frequency difference $\left(\hat{P}_{11}-\hat{P}_{12}\right)$ between cases and controls, and quantified the effect on the type I error rate of Pearson's chi-square test induced by HWD. Additionally, the standard error of relative risk under HWD was shown by Zaykin et al. (2004). In this article, we present a generalized formula for calculating the CI of the allelic odds ratio based on the estimated standard error, which is valid under both HWE and HWD, and then examine the effect of this generalization in a genome-wide association study.

Materials and methods

Derivation of the generalized method of CI calculation

In case-control studies, allelic frequencies are compared between cases and controls. Assuming that two alleles X and x exist at a certain SNP locus, the genotype data are given in a 3 × 2 contingency table as shown in Table 1, the observed frequencies (n_{1j}, n_{2j}, n_{3j}) being distributed as a trinomial distribution Tn ($n_{.j}$; π_{1j}, π_{2j}, π_{3j}) for j=1 (case) and j=2 (control), where (π_{1j}, π_{2j}, π_{3j}) are the population proportions of genotype (XX, Xx, xx), respectively, and $n_{.j}$ (j=1, 2) is the sample size for each population. Of course, $\pi_{1j}+\pi_{2j}+\pi_{3j}=1$ and $n_{1j}+n_{2j}+n_{3j}=n_{.j}$ (j=1, 2).

Let the population proportions of allele X in cases and controls be P_{11} and P_{12}. Then $P_{11}=\pi_{11}+\pi_{21}/2$ and $P_{12}=\pi_{12}+\pi_{22}/2$, and they are estimated as $\hat{P}_{1j}=(2n_{1j}+n_{2j})/(2n_{.j})(j=1,2)$ (Li and Horvitz 1953; Sasieni 1997) in Table 2. The estimator of allelic odds ratio $\psi=\frac{P_{11}(1-P_{12})}{(1-P_{11})P_{12}}$ is given by Eq. 2. (See Appendix.)

$$\hat{\psi}=\frac{\hat{P}_{11}\left(1-\hat{P}_{12}\right)}{\left(1-\hat{P}_{11}\right)\hat{P}_{12}}. \quad (2)$$

When $n_{.1}$ and $n_{.2}$ are large, $\log\hat{\psi}$ is asymptotically distributed as normal with mean and variance given by Eqs. 3 and 4, respectively. (See Appendix.)

$$E\left\{\log\hat{\psi}\right\}\approx\log(\psi). \quad (3)$$

$$V\left\{\log\hat{\psi}\right\}\approx\left(\frac{1}{2n_{.1}P_{11}}+\frac{1}{2n_{.1}(1-P_{11})}\right)(1+F_1)+\left(\frac{1}{2n_{.2}P_{12}}+\frac{1}{2n_{.2}(1-P_{12})}\right)(1+F_2), \quad (4)$$

where F_1 and F_2 are fixation indices of case and control populations, respectively.

Based on the estimated standard error $\mathrm{SE}\left(\log\hat{\psi}\right)$ that is given by Eqs. 5 and 6, an approximate $100(1-\alpha)\%$ CI for ψ is given by Eq. 7. (See Appendix.)

$$\left(\mathrm{SE}\left(\log\hat{\psi}\right)\right)^2=\left(\frac{1}{2n_{11}+n_{21}}+\frac{1}{2n_{31}+n_{21}}\right)\left(1+\hat{F}_1\right)+\left(\frac{1}{2n_{12}+n_{22}}+\frac{1}{2n_{32}+n_{22}}\right)\left(1+\hat{F}_2\right), \quad (5)$$

$$\hat{F}_j=1-\frac{2n_{.j}n_{2j}}{(2n_{1j}+n_{2j})(2n_{3j}+n_{2j})}\quad j=1,2. \quad (6)$$

$$\exp\left(\log\hat{\psi}\pm z_{\alpha/2}\cdot\mathrm{SE}\left(\log\hat{\psi}\right)\right). \quad (7)$$

Table 1 A 3×2 contingency table

Genotype	Case	Control
XX	n_{11}	n_{12}
Xx	n_{21}	n_{22}
xx	n_{31}	n_{32}
Total	$n_{.1}$	$n_{.2}$

Table 2 A 2×2 allele frequency table

Allele	Case	Control
X	$2n_{11}+n_{21}$	$2n_{12}+n_{22}$
x	$2n_{31}+n_{21}$	$2n_{32}+n_{22}$
Total	$2n_{.1}$	$2n_{.2}$

2×2 Contingency table for alleles constructed from Table 1

When HWE is true without doubt, Eq. 5 should be changed to $\hat{F}_1 = \hat{F}_2 = 0$ and then Eq. 7 reduces to Eq. 1, which implies that calculating CI by Eq. 7 is a generalization of the usual method. The essential derivation idea of the generalized method is to introduce the fixation index (F_j) into the population probabilities of genotypes (π_{1j}, π_{2j} and π_{3j}). In actuality, as F_j approaches 0, one automatically arrives at the usual Eq. 1.

Numerical evaluation of the difference of the two formulas

It is obvious from Eq. 5 that the calculated CI is wider in the generalized method than the one in the usual method if $\hat{F}_1>0$ and $\hat{F}_2>0$, while it is narrower if they are less than 0. However, the difference of the two methods should be evaluated numerically, because it is influenced by sampling errors of F_1 and F_2. We evaluated the difference by a numerical calculation of expected upper and lower confidence limits for various values of the fixation indices and sample sizes in the case of P_{11}=0.10 and P_{12}=0.15. In the calculation, we used a normal approximation to the trinomial distribution and the software SAS for computing.

Simulation experiment to examine the influence of generalization

In SNP data analysis, we simultaneously investigate the association between thousands of SNPs and a disease. Some SNPs among them may be under HWD with a distribution of fixation index, while others may be under HWE (F=0). We have to examine the performance of the generalized method for CI calculation, assuming that the fixation indices have a distribution among thousands of SNPs. Consequently, we conducted a Monte Carlo simulation experiment to statistically identify disease-associated SNPs using the decision rule that an association was judged as positive if the calculated CI did not include 1.0.

As the framework of simulation, we set the following conditions referring to the genome-wide association study (Sato et al. 2004):

Condition 1 The total number of SNPs to be examined was set as N=10,000 and the number of disease-associated SNPs (positive SNPs) was set as $N_{.p}$=50, referring to the literature (Sing et al. 1996; Wright et al. 1999; Pharoah et al. 2002; Ponder 2001).

Condition 2 Allelic odds ratio for positive $N_{.p}$ SNPs was ψ=1.5 or 2.0, but ψ=1.0 for the remaining $N-N_{.p}$ SNPs.

Condition 3 The sample size was varied as $n=n_{.1}=n_{.2}$=188, 376 or 752.

Condition 4 The proportion P_{12} of allele X in the control population was a random variable uniformly distributed in unit interval (0.05, 0.95), and P_{11} in the case population was automatically determined by P_{12} through Eq. 20 in Appendix. This condition was set with reference to Fig. 1, to which a uniform distribution is plausible, for the distribution of alleles in the database of Japanese Single Nucleotide Polymorphisms (Haga et al. 2002; Hirakawa et al. 2002). In our genome-scan, we did not include these SNPs with low allele frequency (P_{11}>0.95 or P_{11}<0.05). Note that (π_{1j}, π_{2j}, π_{3j}, j=1, 2) were fixed through Eq. 12 in Appendix when (P_{11}, P_{12}, F) or, equivalently, (P_{11}, ψ, F) was determined.

Condition 5 In a case-group, the fixation index F was specified by a mixed distribution of a constant 0 with probability 1–w and a normal distribution $N(\mu, 0.10^2)$ with probability w, where w=0.02, 0.06 or 0.10, and μ was set as 0.0 (in the null case), 0.2, or 0.4. On the other hand, F was set to 0 for a control group. Note that this condition was set referring to Figs. 2 and 3 taken from a database, Genome Medicine Database of Japan. In order to determine whether normally distributed or not, we showed a quantile–quantile plot in Fig. 2. It showed that the core data reasonably fit a normal distribution, but the tail data do not. Therefore, the distribution of observed F does not have a normal distribution with mean 0. Moreover, around 2% of the larger tail area in Fig. 3 was laid outside the distribution of observed F under the null hypothesis that the

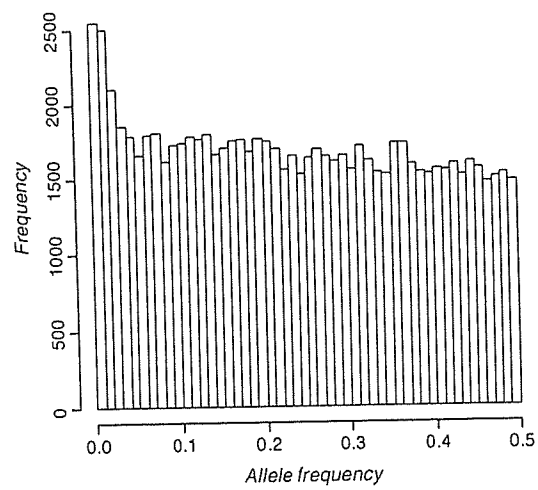

Fig. 1 An example of the minor allele frequency distribution of SNP. The data are from the JSNP database (http://www.snp.ims.u-tokyo.ac.jp/)

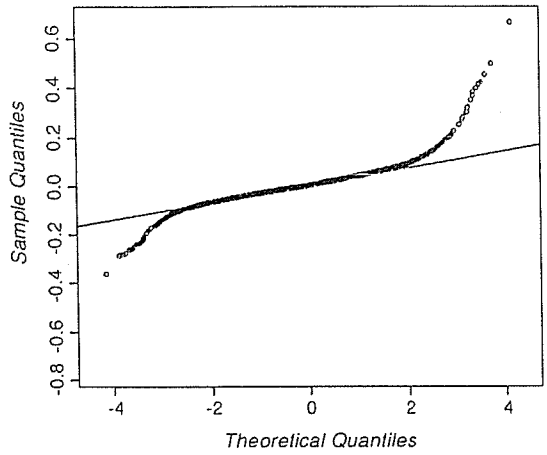

Fig. 2 Quantile–quantile plot for fixation index F in a case-group obtained from Genome Medicine Database of Japan, http://www.gemdbj.nibio.go.jp/dgdb/)

fixation index was equal to 0 and the mean of the outlying values was around 0.2 or more.

Condition 6 The criteria to evaluate the performance of the decision rule were two indicators, positive predictive value R_p and sensitivity R_s, defined by Eqs. 8 and 9 with notations in Table 3.

$$R_p = \frac{N_{TP}}{N_{P.}}, \tag{8}$$

$$R_s = \frac{N_{TP}}{N_{.P}}. \tag{9}$$

Condition 7 The Monte-Carlo simulation to observe R_p and R_s was repeated 1,000 times, and the mean values, together with the mean number of N_{TP} and N_{FP}, were used for comparison of the two methods.

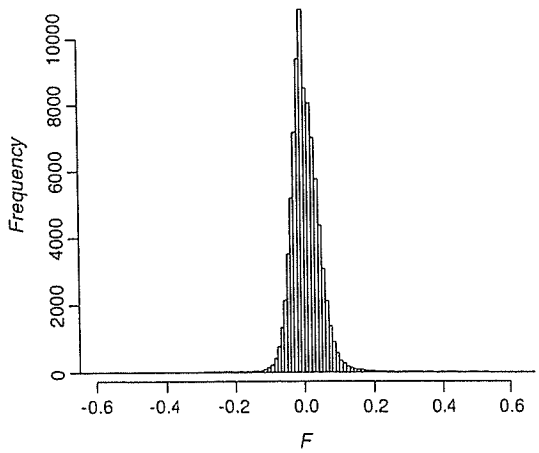

Fig. 3 An example of the frequency distribution of fixation index F in a case-group obtained from Genome Medicine Database of Japan

Table 3 The contingency table for schematic outcomes of a judgment

	True association		Total
	Positive	Negative	
Judgment			
Positive	N_{TP}	N_{FP}	$N_{P.}$
Negative	N_{FN}	N_{TN}	$N - N_{P.}$
Total	$N_{.P}$	$N - N_{.P}$	N

Notation for defining R_p and R_s. Positive predictive value: $R_p = N_{TP}/N_{P.}$, sensitivity: $R_s = N_{TP}/N_{.P}$

Note that $N_{.P}$ was a constant fixed by Condition 1, whereas $N_{P.}$ was a random variable realized as the sum of N_{TP} and N_{FP} in the simulation experiment. Note further that these N_{TP} and N_{FP} have a trade-off relationship depending on the nominal confidence level, but that we fix the nominal confidence level as $1 - \alpha = 0.999$, taking the multiplicity of SNPs into consideration.

The procedure to conduct the simulation experiment was as follows:

Step 1. Assign a set of values to N, $N_{.P}$, ψ, and n according to the above-described conditions.

Step 2. Assign the value $\psi = 1.5$ or 2.0 to the first $N_{.P}$ SNPs and $\psi = 1.0$ to the remaining $N - N_{.P}$ SNPs.

Step 3. Generate 10,000 random numbers of F according to Condition 5 and assign them to 10,000 SNPs.

Step 4. Generate random numbers (n_{11}, n_{21}, n_{31}) and (n_{12}, n_{22}, n_{32}) distributed as $\mathrm{Tn}(n, \pi_{11}, \pi_{21}, \pi_{31})$ and $\mathrm{Tn}(n, \pi_{12}, \pi_{22}, \pi_{32})$, respectively, for each 10,000 SNPs.

Step 5. Calculate CIs using Eq. 1 (usual method) and Eq. 8 (Generalized method) with $\alpha = 0.001$ and calculate N_{TP}, N_{FP}, R_p, and R_s for each 10,000 SNPs.

Step 6. Repeat Steps 1–5 1,000 times and calculate the mean of the realized values.

Step 7. Repeat Steps 1–6, changing parameters ψ in Condition 2, n in Condition 3, and w and μ in Condition 4.

Results

A summarized result of numerical evaluation of the expected confidence limits in a typical case is shown in Table 4 for various values of the fixation index $F = F_1 = F_2$ when the sample size was set at $n_{.1} = n_{.2} = 188$ or