HUMAN MONOCYTOID THP-1 CELL LINE *VERSUS* MONOCYTE-DERIVED HUMAN IMMATURE DENDRITIC CELLS AS *IN VITRO* MODELS FOR PREDICTING THE SENSITISING POTENTIAL OF CHEMICALS

E. BOCCHIETTO, C. PAOLUCCI[1], D. BREDA[1], E. SABBIONI[2] and S.E. BURASTERO[1]

Abich, Tecnoparco, Lago Maggiore, Verbania; [1]San Raffaele Scientific Institute, Department of Biotechnology, Milan; [2]European Centre for the Validation of Alternative Methods, Joint Research Centre, Ispra, Varese, Italy

Received May 25, 2006 – Accepted January 30, 2007

Immature dendritic cells (DCs) modulate differentiation markers following *in vitro* exposure to chemicals generating contact allergies. THP-1 is a monocytoid cell line maintaining some differentiating plasticity. In this study, human DCs and THP-1 cells were compared as *in vitro* models to predict contact sensitisation of chemicals with different sensitising potential. Expression of CD80 and CD86 was assessed by flow cytometry after exposure to subtoxic concentrations of potent (2,4-dinitrochlorobenzene, DNCB and p-phenylendiamine, PPD), strong (thimerosal, TMS), moderate (sodium tetrachloroplatinate, Na_2PtCl_4) sensitising compounds as well as of non-sensitising, irritating sodium dodecyl sulphate (SDS) as compared to a vehicle of sensitising substances (dimethyl sulphoxide, DMSO). Up-regulation of CD86 following *in vitro* incubation of DCs and THP-1 cells with DNCB, PPD, TMS and Na_2PtCl_4, but not with SDS, was observed. The CD80 membrane marker was up-regulated on DCs following *in vitro* incubation with DNCB and PPD, but not with TMS, Na_2PtCl_4 and SDS. On THP-1 cells, only DNCB up-regulated CD80 expression. In conclusion, both the cell line THP-1 and DCs are promising *in vitro* models for assays aiming at predicting the sensitisation potential of chemicals. THP-1 cell line is by far easier to handle and offers relevant advantages from the practical point of view.

Langerhans cells (LCs) of the skin play a crucial role in the induction phase of contact allergy. They bind haptens via major histocompatibility complex (MHC) class II, internalize them (1), and migrate via afferent lymphatics from skin to draining lymph nodes. During migration, they differentiate to mature dendritic cells (DCs) and change morphologically and phenotypically. In the regional lymph nodes, DCs present the antigen to responsive T cells and generate effector T lymphocytes of contact hypersensitivity (2). LCs are poorly represented within epidermal cells and are relatively difficult to isolate in sufficient numbers for *in vitro* studies. In contrast, well-established methods allow generating large numbers of DCs from peripheral blood monocytes (3) which can stimulate naive T cells to effector cells for hypersensitivity reactions (4). Although several authors proposed the use of cultured DCs as an *in vitro* model for contact sensitization (5-6), they still suffer the limitation of a high inter-individual variability in response to chemicals (6-8). As a further possibility, the monocytoid THP-1 stable cell line has been proposed (9-10), but its equivalence to primary DCs has not been established.

Key words: in vitro sensitisation testing, allergotoxicity, dendritic cell line, THP-1 cell line, skin sensitization

Mailing address: Samuele E. Burastero,
San Raffaele Scientific Institute,
58, via Olgettina,
20132 Milano, Italy
Tel: ++39 02 26434730 Fax: ++39 02 26434723
e-mail: s.burastero@hsr.it

0394-6320 (2007)

The aim of the present study is to compare human blood-derived DCs with commercially available THP-1 cell line as *in vitro* assay systems for the prediction of contact sensitization potency of chemicals.

MATERIALS AND METHODS

Chemicals and biochemicals

Chemicals with different sensitization potential selected for the study were: 2,4 dinitrochlorobenzene (DNCB), m.wt.=202,55; p-phenylenediamine (PPD), m.wt.=108,1; thimerosal (TMS), m.wt.=406,33; sodium tetrachloroplatinate (Na_2PtCl_4), m.wt.=168; sodium dodecyl sulphate (SDS), m.wt.=288,38, and dimethyl sulfoxide (DMSO), m.wt=78,13. They were obtained from Sigma Aldrich Italia (Milan, Italy) at the highest purity available.

The sensitization potential of these chemicals was previously established, both on the basis of epidemiological evidence in humans as well as of a validated quantitative *in vivo* assay for the determination of skin sensitization (Local Lymph Node Assay) (11-12). RPMI-1640, fetal calf serum (FCS), antibiotics, glutamine, streptomycin and penicillin were purchased from PBI International (Milano, Italy). Mouse monoclonal antibodies to human CD3, CD80 (IgG1, *k*), CD86 (IgG1, *k*), an isotype-matched monoclonal with irrelevant specificity, recombinant human GM-CSF and IL-4 and Fycoll-Paque were from BD Biosciences Pharmingen (San Diego, CA, USA); Dynabeads M-450 from Dynal Biotech (Oslo, Norway); propidium iodine (PI) and PBS from Sigma Aldrich Italia (Milan, Italy);

THP-1 cell line and monocyte-derived DCs

The human myeloid THP-1 cell line was obtained from the European Collection of Cell Culture (Salisbury, UK), and maintained in RPMI-1640 medium containing 10% FCS and 2 mM glutamine.

DCs were prepared from heparinised blood collected from a vein of the forearm of six healthy donors (30 ml each donor) recruited at the S. Raffaele Hospital (Milan). All subjects were informed on the scope of the study and gave their written consent that was submitted and approved by the local ethics committee (Protocol DS/URC/ER/mm number 177/DG, San Raffaele Scientific Institute). Immature, monocyte-derived DCs were then isolated from peripheral blood as previously described, with some modifications which were introduced in order to verify the plasticity of the differentiation potential of single preparations of DCs (3). Standard gradient separation procedures on Fycoll-Paque were used to isolate peripheral blood mononuclear cells (PBMC) which were then re-suspended in RPMI-1640 containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin ("complete medium"), and allowed to adhere on 6-well plates (Costar, Cambridge, MA) for 1 h at 37°C. In antigen-driven proliferation assays, autologus human serum was used in complete medium. Non-adherent cells were discarded. In order to derive immature DCs, PBMC were then cultured for 7 days in complete medium, in the presence of human GM-CSF (50 ng/ml) and IL-4 (1000 U/ml) (3). Depletion of residual T lymphocytes from immature DCs was carried out by incubating the cell preparations with the anti-CD3 monoclonal antibody (1 µg/10^6 cells) followed by goat anti-mouse IgG–coated Dynabeads M-450. Anti-CD3 bound T cells were removed with a magnet according to manufacturer's instructions. Purified DCs preparations were routinely checked for contaminating lymphocytes by flow cytometry using a fluorescence-activated cell sorter (FACScan plus, Becton Dickinson, Sunnyvale, CA). Residual lymphocytes accounted for less than 1% of total cells. Moreover, DCs preparations were checked for MHC class I and II, CD80, CD83, CD86 and CD1a membrane expression.

As a control for the acquisition of the proper differentiation plasticity, DCs were then incubated for 48 h with (mature DCs) or without (immature DCs) LPS (0.2 µg/ml) and the expression of both CD80 and CD86 membrane markers was assessed by flow cytometry. Only cellular preparations of mature (i.e. LPS-treated) DCs displaying a CD80 and CD86-specific mean fluorescence intensity $\geq$ 2 times than immature DCs were used for further studies.

Exposure of THP-1 and DCs cells to chemicals

Aliquots of fresh aqueous mother solutions of the compounds tested were diluted in complete medium. DNCB was the only chemical that was previously diluted in DMSO (1%) before subsequent

Table I. *Modulation of CD80 and CD86 in DC_s or TPH-1 cells exposed to potent (DNBC, PPD), strong (TMS), moderate (Pt(II)) sensitising and non-sensitising, irritating (SDS) compounds for 48 h compared to DMSO (vehicle for skin sensitisation). Values are expressed as median and inter-quartile ranges (in parenthesis) of NrMFI (For details see Materials and Methods, section "Data presentation and statistical analysis").*

Chemical	Exposure ($\mu g\ ml^{-1}$)	NrMFI[a]							
		CD80				CD86			
		DC_s	p[b]	THP-1	p[b]	DCs	p[b]	THP-1	p[b]
DNCB	0.5	2.23(1.89-3.02)	0.016	1.23(1.23-2.23)	0.031	2.12(2.02-2.23)	0.015	2.09(2.02-2.21)	0.016
PPD	0.5	1.56(1.25-2.13)	0.031	1.23(1.23-1.25)	0.125	2.25(1.89-2.56)	0.031	2.25(2.05-2.56)	0.031
TMS	2	1.56(1.23-2.56)	0.063	2.23(1.89-2.35)	0.063	1.56(1.23-2.35)	0.031	1.49(1.29-2.31)	0.031
Na_2PtCl_4	0.86	1.65(1.45-2.25)	0.063	1.56(1.25-2.25)	0.125	1.55(1.25-2.22)	0.031	1.45(1.23-2.12)	0.031
SDS	0.005(%)	1.02(1.01-1.05)	0.813	1.02(0.99-1.02)	1.000	1.02(0.99-1.02)	1.000	1.02(0.99-1.02)	1.000
DMSO	0.005(%)	1.02(0.99-1.06)	NA	1.02(0.99-1.05)	NA	1.02(0.99-1.02)	-	1.02(0.99-1.03)	NA

progressive dilutions with culture medium.

Dose-effect studies

Both cell types were exposed for 48 h at 37°C and 5% CO_2 to different concentrations of the chemicals ranging from 0.02 to 12.5 µg/ml (DNCB); 0.05-1.0 µg/mL (PPD); 1.0-10 µg/ml (TMS); 0.17-1.6 µg/ml (Na_2PtCl_4), 0.0001-0.005% (SDS) and 0.05% DMSO. Cell death was then determined by flow cytometry as PI incorporation. These experiments allowed the determination of those subtoxic doses (i.e. doses inducing < 50% cell death as assessed by PI-incorporation analysis) of the chemicals, which were used for the subsequent experiment.

Studies with subtoxic concentrations

DCs and TPH-1 cells were exposed in the same experimental conditions to the subtoxic concentrations of the individual chemicals as drawn from the above- mentioned dose-effect studies, and CD80 and CD86 biomarkers were determined by flow cytometry.

Flow cytometric analysis

Cells were harvested from the cultures and counted. Aliquots of 10^5 cells (50 ml PBS+10% FBS) per tube were obtained. Cells were incubated with 10 µl the monoclonal antibody (mAb) conjugated to fluorescein isothiocyanate (FITC) for 10 min on ice and washed twice. The mAb to human CD80-FITC or CD86-FITC was combined with propidium iodide (PI) staining to simultaneously measure the percentage of dead cells. To this aim, a PI working solution (1 ml PI stock solution(1 mg PI/ml water) in 20 ml PI buffer (1 g glucose/l PBS without Ca/Mg)) was added to 10^5 cells. A gate was set on the negative cells in order to exclude the dead cells (Fig. 1, top panels). Only viable cells were included for analyses of CD80 or CD86 expression.

Data presentation and statistical analysis

In each experiment, the results of the phenotypic analysis were expressed as mean fluorescence intensity (MFI) in arbitrary units for the given marker. In order to obtain fluorescence values not susceptible to inter-experiment variability in the setting of the fluorimeter parameters, the relative MFI (rMFI) was extrapolated for each sample, which corresponded to the ratio between the MFI of CD80- or CD86-specific MFI and the MFI of another sample of the same cells reacted with an isotype-matched fluoresceinated mAb with irrelevant specificity (Fig. 1, bottom panels). Finally, in order

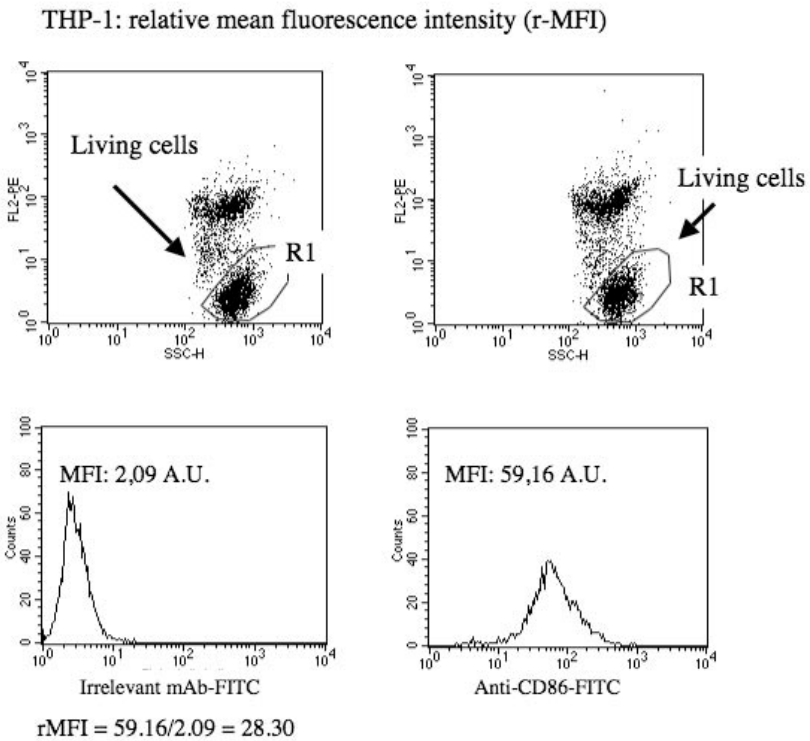

Fig. 1. *Gating strategy for the assessment of CD86 membrane expression on THP-1 cells and calculation of the relative mean fluorescence intensity (rMFI). Flow cytometry profiles are shown of cells reacted with PI and a FITC-conjugated mouse IgG1, k monoclonal antibody, either endowed with irrelevant specificity (left panels) or binding to human CD86 (right panels). The top panels refer to the dot-plot analysis of a physical parameter (SSC, side scatter, on the x-axis) versus PI-staining, which is a marker of cell death (FL-2, on the y-axis). Gate R1 indicate living cells, which were subsequently subjected to histogram analysis (bottom panels). Lower panel histograms depict specific fluorescence intensity (in arbitrary fluorescence channels, indicated as A.U. on the x-axis) of events gated in the respective top panel (number of events are on the y-axis). The rMFI is the ratio between the MFI of CD86-FITC stained sample and the MFI of the sample reacted with the irrelevant monoclonal antibody, as indicated.*

to prevent the effect of different absolute CD80- or CD86-associated fluorescence values in different experiment, due to inter-individual variability of expression or other technicalities, the normalized rMFI (NrMFI) value was extrapolated. NrMFI represented the ratio of the CD80- or CD86-specific rMFI of the sample treated with a given chemical versus the CD80- or CD86-specific rMFI of the untreated cells, respectively. As an example, Fig. 2 shows the results of the parallel evaluation of NrMFI in DCs and THP-1 cells treated with DNCB. Thus, the NrMFI equals the unity in untreated cells in each single experiment, and its variation represents an absolute value of the effect of a given substance on the membrane expression of CD80 or CD86 by DCs or THP-1 cells. In each experiment only the concentration yielding the highest difference in NrMFI as compared to vehicle DMSO was used for the statistical analysis.

Since the relatively small sample size did not allow to establish whether data were normally distributed or not, the Student t-test was not used. Instead, the Wilcoxon unmatched pair signed-rank test was utilized to evaluate whether the value of NrMFI of the DMSO-(vehicle) treated sample of one individual (in the case of DCs) or of one test (in the case of THP-1) versus the value of NrMFI of the given chemical treated-sample differed in size. Values of $p < 0.05$ were considered as significant.

RESULTS

Dose-effect studies

Fig. 3 shows the results of the dose-effect studies

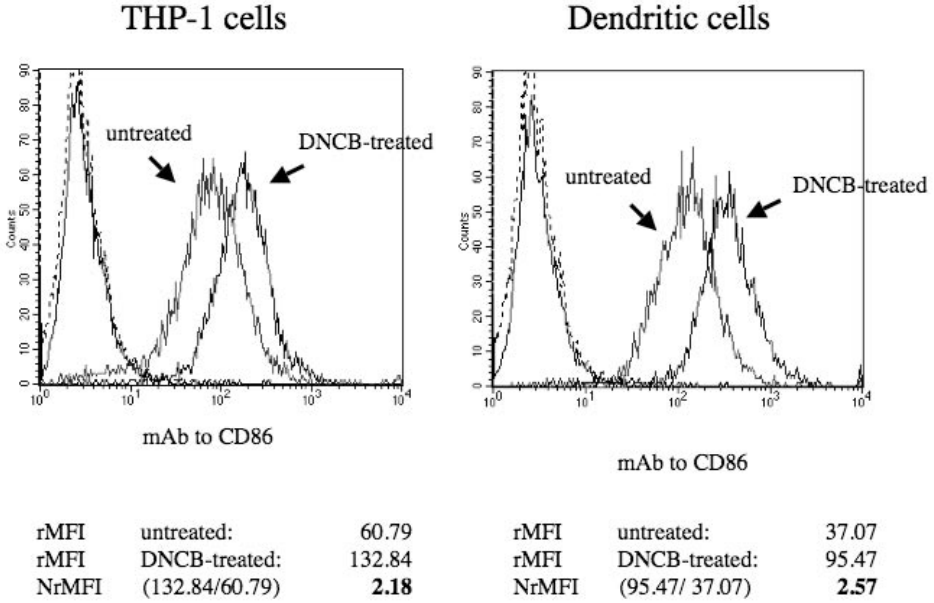

Fig. 2. *Modulation of CD86 membrane expression by DCs (left panel) and THP-1 cells (right panel) upon 48 hr treatment with DNFB: calculation of the normalized rMFI (NrMFI). Histogram analysis shows fluorescence intensity (expressed as mean fluorescence intensity, MFI, on the x-axis) of untreated and DNFB-treated cells, respectively, as indicated by the arrows. Number of events is on the y-axis. The profiles of fluorescence intensity of the untreated (continuous line) and treated sample (dotted line) when reacted with an irrelevant monoclonal antibody are also depicted, which allowed the extrapolation of the relative MFI (rMFI). The formula used for the calculation of the normalized rMFI (N-rMFI) is indicated.*

concerning the exposure of DCs and TPH-1 cells for 48 h to different concentrations (0.002-12.5 µg/ml) of the potent sensitizing compound DNBC, and of a non-sensitizing, irritating agent (SDS, 0.0001-0.0015%).

The following conclusions can be drawn:

(i) At exposure as low as 2 µg/ml the cell death was around 30% as determined by PI+. Concentrations of 10 µg/ml and 12.5 µg/ml induced 50% (IC_{50}) and complete cell mortality, respectively. A concentration of 0.5 µg/ml of DNBC was drawn as subtoxic concentration for the subsequent study of CD80 and CD86 expression compared to DMSO. In both cell lines the highest CD80 and CD86 expression was achieved at 0.5 µg/ml of DNCB.

(ii) At exposure as low as 0.005% SDS the cell death was of the order of 30% while a complete cell mortality was observed at 0.015% exposure. A subtoxic SDS concentration of 0.0075% was drawn for the subsequent study of CD80 and CD86 expression compared to DMSO. CD80 and CD86 expression was constant at subtoxic as well as at other SDS tested concentrations.

From the corresponding graphical representations of the other chemicals investigated (PPD, TMS, Na_2PtCl_4, figures not shown) the following subtoxic concentrations were drawn for the subsequent study of CD80 and CD86 expression compared to DMSO: 0.5 µg/ml (PPD); 2 µg/ml (TMS) and 0.84 µg/ml (Na_2PtCl_4).

Modulating expression of CD80 and CD86 in DCs cells

Table I summarizes the results of the CD80 and CD86 expression in DCs cells exposed for 48 h to selected subtoxic concentrations of potent (DNCB and PPD), strong (TMS), moderate (Na_2PtCl_4) sensitizing- and of the non-sensitizing, irritating (SDS) compounds compared to a vehicle for skin sensitization testing (DMSO). Values are expressed as CD80 and CD86-specific NrMFI. The following conclusions can be drawn:

(i) incubation with 0.5 µg/ml of DNCB allows a

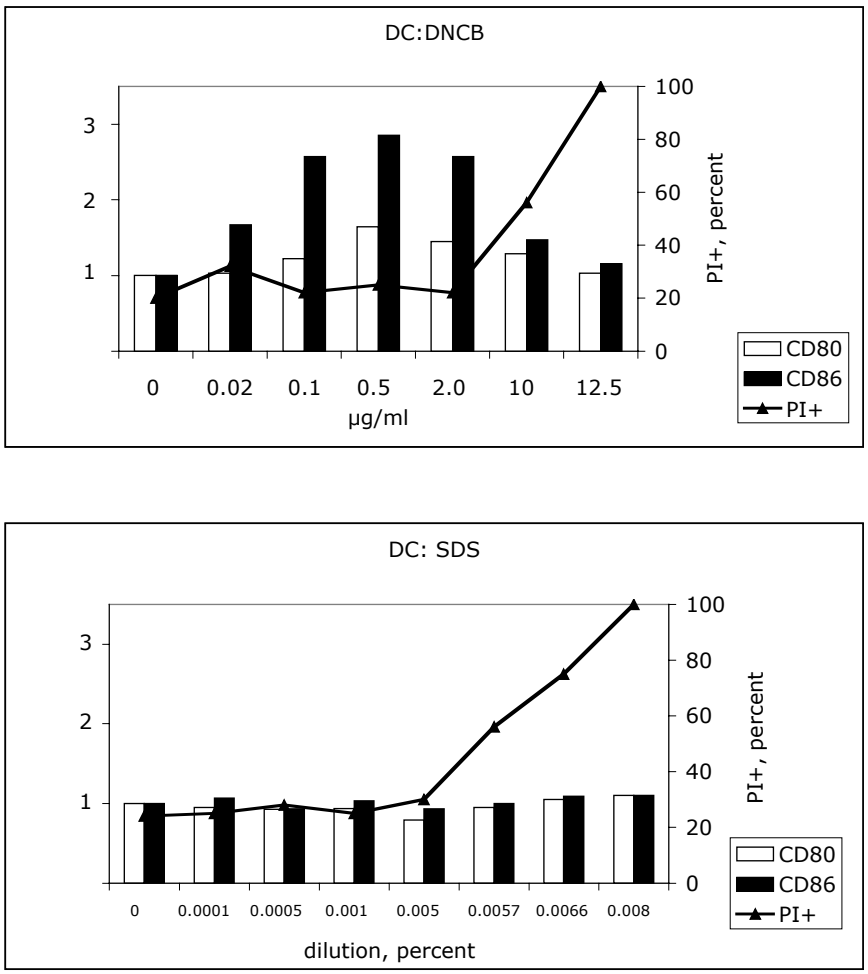

Fig. 3. *Dose response analysis of N-rMFI values (on the left y-axis of each panel) for CD80 and CD86 (white and grey columns, respectively) following treatment of DCs (top panels) and THP-1 cells (bottom panels) with the indicated substance at the concentration shown on the x-axis. The values of the proportions of dead cells expressed as percentage (on the right y-axis of each panel) of propidium iodine (PI) stained cells at each concentration are indicated by the filled triangles.*

significant increase of the median values of both CD80 and CD86-specific NrMFI as compared to DMSO (p=00.016 and 0.015 respectively). Similar results were observed for the exposure to 0.5 µg/ml of the second potent sensitizing chemical (PPD, p=0.031 for both CD80 and CD86).

(ii) exposure to 2 µg/ml of TMS did not significantly increase CD80 (p=0.063) but increased CD86 (p=0.031) expression.

(iii) incubation with 0.84 µg/ml of Na_2PtCl_4 led to similar expression patterns to those obtained from the exposure to TMS: no significant increase of CD80 (p=0.063) and up regulation of CD86 (p=0.031).

(iv) nor did 0.0075% of SDS significantly change either CD80 or CD86 expression (p=0.813 and 1, respectively).

Modulating expression of CD80 and CD86 in THP-1 cells

Table I also reports the corresponding data obtained on THP-1 cells:

(i) exposure of the cells to 0.5 µg/ml of DNCB led to an increase in both CD80 and CD86 expression (p=0.031 and 0.016 respectively)

(ii) incubation with 0.5 µg/ml of PPD resulted in an increase of CD86 (p=0.031) but not of CD80 (p=0.125) markers
(iii) cells exposed to 2 µg/ml of TMS did not change the CD80 expression (p=0.063) whereas CD86 biomarker was up regulated (p=0.031)
(iv) concentration of 0.84 µg/ml of Na2PtCl4 did not vary the expression of CD80 membrane marker (p=0.125) but significantly increased CD86 expression (p=0.031)
(v) 0.0075% of SDS in culture medium did not affect the modulation of both CD80 and CD86 (p=1).

DISCUSSION

DCs cells are among the best candidates for designing *in vitro* alternative approaches to animal test methods aimed at identifying skin sensitising chemicals (6, 8, 13-14). The recent ECVAM Workshop 51 Report reviewed the current status of the use of cultured DCs as a tool for the predictive identification of skin sensitisation hazard (15). The same Report also suggests that stable cell lines should be considered as potential useful tools in this context, warranting further investigations on human myeloid undifferentiated U-937 and THP-1 cell lines (15). The present work specifically compares DCs and TPH-1 cells as tools for the *in vitro* prediction of allergenicity. We tested chemicals with different degrees of sensitizing potential over wide concentration ranges, and adopted several precautions to limit the effect of inter-individual and batch-to-batch variability of DCs.

Our results confirm the possibility of using the expression of selected co-stimulatory molecules (CD80 and CD86) on DCs and THP-1 cells as virtually equivalent endpoints in testing for prediction of skin sensitization of chemicals, and also highlight specific limitations. Although THP-1 cells may have a relatively heterogeneous composition in terms of the differentiation stage of its cellular components, as compared to DCs they have the advantage of being less expensive, not subjected to limitations in availability and much easier to be cultured. Our data support the notion that, in a tiered *in vitro* testing strategy to predict skin sensitisation hazard of chemicals, a THP-1 pre-screening should eliminate potent, strong and moderate sensitising compounds before further predictive DCs testing. Additional investigations on a larger number of chemicals with quantitatively known allergenicity are needed to clarify this issue. In particular, two topics should be addressed. The first one concerns the extent of the metabolic activity of DCs and THP-1 cells and whether this is sufficient for the routine identification of pro-haptens (15). The second concerns the effect of speciation on the biological response which is measured *in vitro* (16-17). How different inorganic and metallorganic species of an individual element influences the expression of membrane biomarkers is of fundamental importance for a full estimation of the performance of the cellular models for assessing the skin sensitisation hazard of chemicals.

REFERENCES

1. **Kimber I., M. Cumberbatch, C.J. Betts and R.J. Dearman.** 2004. Dendritic cells and skin sensitisation hazard assessment. *Toxicol. In Vitro 18:195.*
2. **Cumberbatch M., R.J. Dearman and I. Kimber.** 1997. Interleukin 1 beta and the stimulation of Langerhans cell migration: comparisons with tumour necrosis factor alpha. *Arch. Dermatol. Res. 289:277.*
3. **Paolucci C., S.E. Burastero, P. Rovere-Querini, C. De Palma, S. Falcone, C. Perrotta, A. Capobianco, A.A. Manfredi and E. Clementi.** 2003. Synergism of nitric oxide and maturation signals on human dendritic cells occurs through a cyclic GMP-dependent pathway. *J. Leukoc. Biol. 73:253.*
4. **Cella M., F. Sallusto and A. Lanzavecchia.** 1997. Origin, maturation and antigen presenting function of dendritic cells. *Curr. Opin. Immunol. 9:10.*
5. **Aeby P. and M. Bracher.** 1999. Evaluation of CD86 modulation and IL-1 beta gene expression on cultured dendritic-like cells as predictive endpoints for contact sensitizers. *ATLA 27:359.*
6. **Tuschl H., R. Kovac and E. Weber.** 2000. The expression of surface markers on dendritic cells as indicators for the sensitizing potential of chemicals. *Toxicol. In Vitro 14:541*.
7. **Pichowski J.S., M. Cumberbatch, R.J. Dearman, D.A. Basketter and I. Kimber.** 2001. Allergen-induced changes in interleukin 1 beta (IL-1 beta) mRNA expression by human blood-derived dendritic cells: inter-individual differences and relevance for sensitization testing. *J. Appl. Toxicol. 21:115*.

8. **Aiba S., A. Terunuma, H. Manome and H. Tagami.** 1997. Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur. J. Immunol. 27:3031.*
9. **Mizuashi M., T. Ohtani, S. Nakagawa and S. Aiba.** 2005. Redox imbalance induced by contact sensitizers triggers the maturation of dendritic cells. *J. Invest. Dermatol. 124:579.*
10. **Yoshida Y., H. Sakaguchi, Y. Ito, M. Okuda and H. Suzuki.** 2003. Evaluation of the skin sensitization potential of chemicals using expression of co-stimulatory molecules, CD54 and CD86, on the naive THP-1 cell line. *Toxicol. In Vitro 17:221.*
11. **Basketter D.A., K.E. Andersen, C. Liden, H. Van Loveren, A. Boman, I. Kimber, K. Alanko and E. Berggren.** 2005. Evaluation of the skin sensitizing potency of chemicals by using the existing methods and considerations of relevance for elicitation. *Contact Dermatitis 52:39*.
12. **Gerberick G.F., C.A. Ryan, P.S. Kern, H. Schlatter, R.J. Dearman, I. Kimber, G.Y. Patlewicz and D.A. Basketter.** 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis. 16:157.*
13. **Arrighi J.F., M. Rebsamen, F. Rousset, V. Kindler and C. Hauser.** 2001. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. *J. Immunol. 166:3837.*
14. **De Smedt A.C., R.L. Van Den Heuvel, N. Zwi Berneman and G.E. Schoeters.** 2001. Modulation of phenotype, cytokine production and stimulatory function of CD34+-derived DC by NiCl(2) and SDS. *Toxicol. In Vitro 15:319.*
15. **Casati S., P. Aeby, D.A. Basketter, A. Cavani, A. Gennari, G.F. Gerberick, P. Griem, T. Hartung, I. Kimber, J.P. Lepoittevin, B.J. Meade, M. Pallardy, N. Rougier, F. Rousset, G. Rubinstenn, F. Sallusto, G.R. Verheyen and V. Zuang.** 2005. Dendritic cells as a tool for the predictive identification of skin sensitisation hazard. *Altern. Lab. Anim. 33:47.*
16. **Mazzotti F., E. Sabbioni, J. Ponti, M. Ghiani, S. Fortaner and G.L. Rossi.** 2002. *In vitro* setting of dose-effect relationships of 32 metal compounds in the Balb/3T3 cell line, as a basis for predicting their carcinogenic potential. *Altern. Lab. Anim. 30:209.*
17. **Di Gioacchino M., L. Di Giampaolo, N. Verna, M. Reale, M.B. Di Sciascio, A.R. Volpe, M. Carmignani, J. Ponti, R. Paganelli, E. Sabbioni and P. Boscolo.** 2004. *In vitro* effects of platinum compounds on lymphocyte proliferation and cytokine release. *Ann. Clin. Lab. Sci. 34:195.*