NEW APPROACH FOR CHEMICAL SENSITIZING POTENTIAL ASSESSMENT USING THP-1 and NCTC 2544 CO-CULTURE

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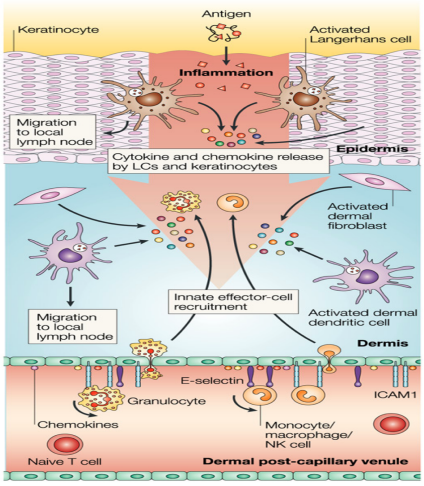
INTRODUCTION

It has been demonstrated that dendritic cells which play a crucial role in the immunity, can be activated by certain chemicals and therefore, have become a promising tool for detection of contact allergens (Casati et al. 2005, Kimber et al., 2001).Several cell lines, suggested as surrogates for dendritic cells, are currently explored for their potential to predict sensitizers : several studies (Sakaguchi H. 2010, Ashikaga T. 2006) demonstrated that human myeloid cell lines such as THP-1 is good surrogate for DC .The property of the monocytic cell line THP-1 (h-CLAT) to discriminate between skin sensitizers and non-sensitizers has been developed through a interlaboratory validation conducted by 5 labs (P&G, Shiseido, Kao, Henkel and L'Oreal): the determination of the surface markers CD86 and CD54 were used for the discrimination between sensitizers and non-sensitizers (Sakaguchi et al., 2007, 2010). The test method is undergoing a prevalidation/validation process at ECVAM (Ashikaga et al., 2010). However a direct approach of the allergic response based on DC studies globally lacks of the contribution of keratinocytes that have a pivotal role in the allergic reaction via the release of solubles mediators : haptens cause activation of keratinocytes resulting in the rapid production of a whole array of inflammatory cytokines (e.g. IL-1β, IL-6, and TNF-a,) and chemokines involved in the recruitment and activation of DC Furthermore the presence of a regular Stratum Corneum is a limiting factor to skin penetration able to better address the question to identify the potency of the molecule. We hypothesize that cross-talks between keratinocytes and DC are mandatory for an efficient uptake and processing of haptens by skin DC leading to sensitization. Recently, several studies pointed out that a variety of skin sensitizers significantly induce CD54, CD86, and CD40 expression on the human monocytic leukaemia cell line THP-1, while non-sensitizers do not (Ashikaga et al., 2002; Yoshida et al., 2003; Miyazawa et al., 2007).

Aim of this study was first to confirm in our hand the results of the THP-1 model on a serie of reference chemicals(1-CHLORO-2,4-DINITRO-BENZENE,HYDROQUINONE,ETHYLENEDIAMINE,HEXYL-CINNAMALDEHYDE, LACTIC ACID) with a prediction model based on changes in DC transcriptional activity of selected biomarkers. Then in order to take into account the Kc contribution to skin sensitization potential a KC-DC surrogate co-culture (NCTC-2544 human keratinocytes and THP-1 cell line) has been used as a relevant model to study the cross-talk between keratinocytes and dendritic cell after the contact with a serie of skin sensitizers.
This loose-fit co-culture develops into an allergen-sensitive system consisting of keratinocytes (KC) and of mobile DC-related cells.



MATERIAL AND METHODS

THP-1 cell culture : THP-1 cells, Acute Monocytic Leukemia, are supplied by the American Type Culture Collection (ATCC Cat. N. TIB-202) and was cultured in RPMI 1640 medium supplemented with:2 mM Glutamine,10 mM HEPES,1 mM Sodium Pyruvate,4500 mg/L Glucose,1500mg/L Sodium Bicarbonate,0,05 mM 2-beta-mercaptorthanol,1% penicillin-streptomycin (Pen-Strep), and 10% fetal bovine serum (FBS).
NCTC-2544 cell culture : NCTC-2544, skin keratinocytes, are supplied by the Cell Bank, Interlab Vell Line Collection (ICLC) and was cultured in EMEM medium supplemented with 2 mM Glutamine, 1% penicillin-streptomycin (Pen-Strep), 1% non-essential amino acids and 10% fetal bovine serum (FBS)

THP-1 /NCTC-2544 : CO-CULTURE: THP-1 cells were maintained under standard culture conditions at a temperature of 37 °C in 5% humidified CO2. Cell densities of 0.1 till 0.5 x 10^6 cells/mL will be applied for subculture: medium renewal every 2 or 3 days.Cells were in suspension.NCTC-2544, adherent cells was grown at a temperature of 37 °C in 5% humidified CO2 till confluence.

EXPERIMENTAL DESIGNS

The concentration inducing 75% cell viability was selected for the skin sensitization test (value indicated in Table I).The results of the MTT at the two exposures were not significantly different and the time 24h was preferred for the sensitization studies.At the end of the exposure time, total RNA was extracted and used for real-time quantitative RT-PCR with Taqman® assay reagents for CD86 and CD54 , and IL-8 as skin sensitization biomarkers, beta actin as the endogenous control. Data were represented as Relative Quantification data (RQ).

DATA ACQUISITION AND ACCEPTANCE CRITERIA

Fluorescence data of the RT-PCR generated by the thermocycler ABI PRISM 7500, are collected by the internal software SDS 1.3.1.Because each cycle in the PCR reaction corresponds to a 2-fold increase in PCR product, a difference of one in threshold cycle number represents a 2-fold difference in the expression of a particular gene compared to the calibrator sample and can be considered as significant. 95% of confidence level is used by the software to calculate the errors.

PROPOSED PREDICTION MODEL

The prediction model is based on the gene expression results (relative quantification data=RQ) of three genes after 24h exposure on THP-1:

24H RESULTS IN VITRO CD86, CD54, IL8 GENES EXPRESSION	CLASS	CLASSIFICATION RESULTS ON THP-1 MONOLAYER
RQ VALUES OF THE THREE GENES ≥ 2	EXTREME SENSITIZER	DNCB
RQ VALUES OF AT LEAST ONE OF THE THREE GENES ≥ 2	STRONG SENSITIZER	HQ
RQ VALUES: 0 < RQ < 2	NON SENSITIZER	LA

STEP I: CYTOTOXICITY ASSAY ON THP-1

The non cytotoxic dose on THP-1 cell line was assessed by MTT test: each substance was tested in triplicate at three different concentrations for 24h or 4h followed by a post incubation of 20h.

STEP II A: SKIN SENSITIZATION TEST ON THP-1 ALONE

Cells were seeded at 1 · 10^6 cells/mL in a 24-well plate and cultured with chemicals in triplicate for 24h.

RESULTS Fig.1

By using the results of gene expression of 3 genes (CD86, CD54 and Il-8) it has been possible to correctly classify the extreme (DNCB) and strong (HQ) sensitizers. Identification of extreme sensitizer is possible according the 3 up-regulated gene expression studied and identification of strong sensitizer is possible according to the IL-8 gene expression.

STEP II B: SKIN SENSITIZATION TEST : CO-CULTURE THP-1 AND KERATINOCYTES NCTC-2544

NCTC-2544 will be seeded in a 24-well plate and let growth till confluence. After reaching confluence, THP-1 cells will be seeded at 1 · 10^6 cells/mL in the same plate in RPMI-1640 medium and treated with selected concentration of chemicals in triplicate for 24h (values reported in Table II) .

RESULTS Fig. 2

The results of the transcriptional study on THP-1 cells in the co-culture model study showed that identification of strong sensitizer is possible according to the IL-8 gene expression .However in our experimental conditions it has not been possible to discriminate the other reference molecules. There is a need to optimize the experimental conditions by exploring different issues: lack of sensitivity due to the dose or to the exposure, to phagocytosis of haptens by keratinocytes.

TABLE I

REFERENCE CHEMICALS	LNNA POTENCY CATEGORY	CONCENTRATION FOR MTT TEST µg/mL	24H	4H+20H
1-CHLORO-2,4-DINITRO-BENZENE	EXTREME SENSITIZER	1	158,34	138,76
		4	79,15	108,59
		10	22,23	132,78
HYDROQUINONE	STRONG SENSITIZER	10	118,57	49,13
		30	72,205	77,196
		60	72,133	47,678
ETHYLENEDIAMINE	MODERATE SENSITIZER	100	118,57	159,08
		200	72,205	104,57
		300	72,133	107,61
HEXYLCINNAMALDEHYDE	WEAK SENSITIZER	5	134,71	100,68
		25	131,77	100,04
		40	163,96	102,53
LACTIC ACID	NON SENSITIZER	10	138,76	99,497
		100	198,59	79,425
		1000	132,78	91,776

TABLE II

REFERENCE CHEMICALS	UNIVOCAL CODE	LNNA POTENCY CATEGORY	CONCENTRATION FOR THE SENSITIZATION TEST
1-CHLORO-2,4-DINITRO-BENZENE	DNCB	EXTREME SENSITIZER	3 µg/mL
HYDROQUINONE	HQ	STRONG SENSITIZER	30 µg/mL
ETHYLENEDIAMINE	ED	MODERATE SENSITIZER	200 µg/mL
HEXYLCINNAMALDEHYDE	HCA	WEAK SENSITIZER	25 µg/mL
LACTIC ACID	LA	NON SENSITIZER	100 µg/mL

For DNCB and HCA DMSO was used at a total concentration lower than 0.2 % in the medium.

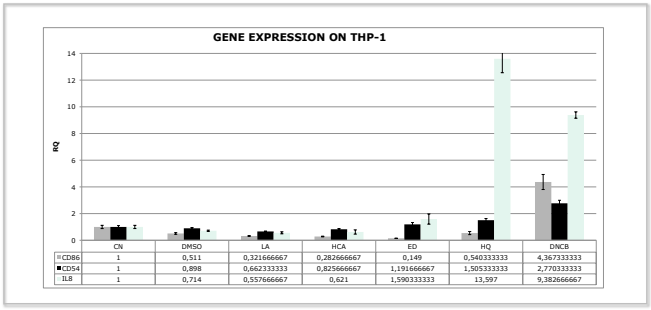


FIG.1

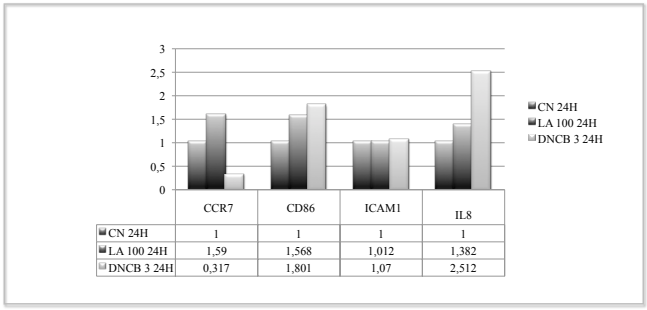


FIG.2

CONCLUSION

The results reported in this study have shown that the THP-1 cell line model was able to discriminate skin sensitizers from non-sensitizers by using gene expression of relevant Biomarkers and has confirmed the results obtained by cytometric analysis currently under validation process. The new approach based on the transcriptional modification following a contact with a chemical provides a new screening alternative method to existing assays. The loose-fit co-culture model of DC cell line co-cultured with Kc cell line demands further investigation to optimize the protocol and to reach a deeper understanding of the Kc influence. Currently we are studying the influence of Inflammatory mediators synthetized by cutaneous epithelial cells that are major actors in ACD and their effect are not taken into account in existing assays.
The final aim of this research project will be to develop a reconstructed epidermis/DC co-culture assay more relevant to the early events of the pathophysiology of ACD in order to increase the sensitivity (to discriminate weak allergens) enables testing of topically applied finished products.This approach will take into account the bioavailabilty of chemicals, the absorption and also skin metabolism. This assay could be a new and ethical tool in a tiered test strategy used to assess the skin sensitization potential and could be complementary used depending on respective specific applicability domains.

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