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Activation of U937 Cells by Contact Sensitizers: CD86 Expression is Independent of Apoptosis

Nadège Ade and Silvia Martinozzi-Teissier

L'Oréal Recherche, Aulnay-sous-Bois, France

Marc Pallardy

Laboratoire d'Immunotoxicologie, INSERM UMR 749, Faculté de Pharmacie, Châtenay-Malabry, France

Françoise Rousset

L'Oréal Recherche, Aulnay-sous-Bois, France

Among the different phenotypic changes induced by contact sensitizers in dendritic cells and myeloid cell lines, CD86 appears to be a consensus marker, since constantly described as systematically up-regulated. To evaluate the robustness of this marker, interference of cytotoxicity on CD86 expression was investigated in U937 myelomonocytic cell line. In this study, cytotoxicity observed at 48 hr (reading-time for CD86 expression) after treatment with DNCB, NiSO₄ and PPD was shown to result from apoptosis taking place at earlier time points. This allergen-induced apoptosis was at least partly caspase-dependent as demonstrated by caspase-3 activation in response to DNCB and NiSO₄ and inhibition of DNCB-induced apoptosis by Z-VAD-fmk. Inhibition of apoptosis did not modify the stimulation index of CD86 expression in DNCB-treated cells, indicating that apoptosis did not interfere with up-regulation of CD86 expression. In addition, similar CD86 expression level was found in DNCB-treated cells whether calculated from the whole non-necrotic cell population including apoptotic cells or from viable non-apoptotic cell population only. Altogether, these results brought evidence that the presence of cells engaged in death process are not a confusing factor for CD86 expression in response to contact sensitizers. They also pointed out apoptosis as another possible key marker of cellular response to contact sensitizers.

Keywords contact dermatitis, CD86, apoptosis, U937, *in vitro* alternatives to animal testing

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Abbreviations: CD86-S.I., CD86 stimulation index; A/N, apoptosis/necrosis; DC, dendritic cell; DNCB, 1-chloro-2,4-dinitrobenzene; MoDC, monocyte-derived dendritic cell; NiSO₄, Nickel sulfate; PI, propidium iodide; PPD, para-phenylenediamine.

Address correspondence to Françoise Rousset, L'Oréal Recherche, 1, avenue Eugène Schueller, BP22, 93600 Aulnay-sous-Bois, France; e-mail: frousset@rd.loreal.com

INTRODUCTION

Allergic contact dermatitis is a cell-mediated immune response to small molecular weight chemicals, also called haptens that come in contact with and penetrate the skin inducing the activation of epidermal Langerhans cells. Once these specialized dendritic cells (DC) have taken up the hapten, they migrate from epidermis to the draining lymph node where they present antigen to T-lymphocytes to drive an adaptive immune response (Kripke et al., 1990). It is relevant that DC may play a crucial role in sensing danger signals in the local microenvironment (Gallucci and Matzinger, 2001) and particularly during the induction phase of delayed type hypersensitivity.

It has been previously demonstrated that *in vitro* treatment with contact sensitizers can induce phenotypic changes in monocyte-derived DC (MoDC) (Aiba et al., 1997, 2003; Aiba, and Tagami, 1999; Coutant et al., 1999; Rousset et al., 2000; Tuschl et al., 2000; Arrighi et al., 2001; Tuschl and Kovac, 2001; Aebly et al., 2004; Mizuashi et al., 2005), in CD34⁺-derived DC (Rougier et al., 2000; Boislevy et al., 2004; De Smedt et al., 2005), as well as in human myeloid cell lines (Ashikaga et al., 2002; Rousset et al., 2002; Yoshida et al., 2003; Azam et al., 2006; Python et al., submitted for publication). These phenotypic changes include up-regulation of different co-stimulatory and maturation molecules.

Among these, CD86 expression appears to be a consensus marker for contact sensitizers, since it has been most constantly reported whatever the cellular models previously described, whereas neither B-lymphocytes (personal observation) nor macrophages were responders (Manome et al., 1999). Some authors have reported that up-regulation of CD86 induced by contact sensitizers was often accompanied by an increased cytotoxicity (Hulette et al., 2005; Sakaguchi et al., 2005; Straube et al., 2005). By testing different sensitizers and non sensitizers, Hulette et al. (2005) concluded that only contact allergens

reproducibly potentiated CD86 expression when used at slight to moderate cytotoxic concentration. Taken advantage of this first analysis on the specificity of the CD86 detection, and to validate the robustness of an *in vitro* predictive test for the assessment of skin sensitization potential based on the CD86 expression, it then appeared necessary to better characterize the relationship, if any, between CD86 up-regulation and cytotoxicity.

Apoptosis and necrosis are the major mechanisms leading to cell death. Necrosis has been described as a passive and accidental cell death resulting from environmental disturbances whereas apoptosis is a programmed cell death, characterized by morphological and biochemical changes including internucleosomal DNA cleavage and randomization of the distribution of phosphatidylserine between the inner and the outer leaflets of the plasma membrane (Fink et al., 2005). The group of Aiba et al. (Aiba and Tagami, 1999; Manome et al., 1999) previously reported that NiCl₂ and CoCl₂ induced apoptosis in MoDC.

In the present study, we focused our work on U937 cells since we and others had shown that this type of cells responded to contact sensitizers dose-dependently by up-regulating CD86 expression (Rousset et al., 2002; Python et al., submitted for publication). To analyze the influence of cytotoxicity induced by two sensitizers, NiSO₄ and DNCB, in the up-regulation of CD86 expression in U937 cells, we first characterized the nature of cell death provoked by these molecules (apoptosis/necrosis). We then checked whether apoptosis induced by these sensitizers was not a confusing factor when measuring changes in CD86 surface marker expression on U937 cells.

MATERIALS AND METHODS

Materials

Nickel sulfate (NiSO₄), 1-chloro-2,4-dinitrobenzene (DNCB), para-phenylenediamine (pPD) and sodium dodecyl-sulfate (SDS) were purchased from Sigma (St Louis, MO). NiSO₄, pPD, and SDS were dissolved in culture medium. DNCB was made soluble in dimethylsulfoxide (DMSO, Sigma) at 0.4% final concentration. The peptide inhibitor Z-VAD-fmk (Z-Val-Ala-Asp (*O*-methyl)-fluoromethylketone) was purchased from R&D Systems (Abington, UK), made soluble as 20 mM stock solution in DMSO, and stored at -20°C. PBS (without Ca⁺⁺ and Mg⁺⁺) was provided by Biowhittaker (Verviers, Belgium). Propidium iodide (PI) and Annexin V-FITC were from R&D Systems. 7-AAD for cell necrosis staining was from Beckman CoulterTM (Marseille, France). Fluorescein isothiocyanate (FITC)-or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs), anti-human-CD86 (FUN-1), and isotypic control IgG, were purchased from Pharmingen (San Diego, CA).

Cell Culture

U937, a human myelomonocytic cell line, was obtained from the American Type Culture Collection (Rockville, MD). Cells

were cultured at 37°C under a humidified 5% CO₂ / 95% air atmosphere in RPMI 1640 supplemented with 100 U/ml penicillin, 500 µg/ml streptomycin, 2 mM L-glutamine (Sigma) and 10 % fetal bovine serum (FBS) (BioWest, France). On the day of experiment, cells were seeded in 96-well culture plates (Nunc, Roskilde, Denmark) at a density of 1×10^6 cells/ml (200 µl/well) with or without test chemicals.

Determination of CD86 Expression

Cell staining was performed using mouse mAb FITC- or PE-anti-human-CD86. Briefly, cells were washed in cold PBS (containing 5% FBS) and stained with antibodies in the dark at 4°C for 30 min, washed twice with cold PBS (containing 5% FBS), once with PBS without FBS and resuspended in PBS. Cells were then analyzed using CellQuest Software (Becton Dickinson, San Jose, CA) based on a collection of 10,000 cells with a FACScan flow cytometer (Becton Dickinson). Necrotic cells were gated out after staining with 50 µg/ml PI (FL-3 channel) solution. The percentage of CD86 positive cells was determined according to the IgG₁ isotype control. The percentage of CD86 positive cells in untreated cells was constantly around 3%. A CD86 Stimulation Index (CD86-S.I.) was calculated as follows: [% of CD86 positive cells in treated cells]/[% of CD86 positive cells in untreated cells] × 100.

Detection of Apoptosis with Annexin V Staining

Phosphatidylserine externalization during apoptosis was studied using double staining with Annexin V-FITC and PI according to the manufacturer instructions (R&D Systems). Briefly, cells were washed once with cold PBS and then resuspended in buffer containing HEPES with 1.8 mM CaCl₂ at a concentration of 4×10^5 cells/100 µl. One µl of Annexin V-FITC (25 µg/ml) and 10 µl of PI (50 µg/ml in PBS) were added to 100 µl buffer. Cells were then resuspended in this mixture and incubated for 15 min at room temperature in the dark. Following incubation, 400 µl of the buffer were added to each tube. Unlabelled cells, Annexin V-FITC-stained cells (FL-1 channel), and PI-stained cells (FL-2 channel) were run first to optimize the settings. Then, 10,000 cells were analyzed using a FACScan flow cytometer and classified as viable (Annexin V⁻/PI⁻, bottom left quadrant), apoptotic (Annexin V⁺/PI⁻, bottom right quadrant) or necrotic/late apoptotic (Annexin V⁺/PI⁺, upper right) based on regions drawn on the dot plots.

Annexin V/7-AAD/CD86 Co-staining

Firstly, cells were stained with anti-CD86-PE mAb (FL-2 channel) as described previously. Then cells were stained with Annexin V-FITC (FL-1 channel) and 7-AAD (FL-3 channel). For FACS analysis, necrotic/dead cells (7-AAD⁺ cells, FL-3 channel) were gated out by exclusion of 50 µg/ml 7-AAD solution. CD86 expression was determined by gating the whole cells (Annexin V⁻ and Annexin V⁺/7-AAD⁻, i.e., viable and

apoptotic cells) and compared to the expression of CD86 expression determined by gating only viable cells (Annexin V⁻/7-AAD⁻).

Quantitative Evaluation of Human Active Caspase-3 in Cell Extracts

Caspase-3 activity was assessed with an immunoassay kit purchased from R&D Systems. Briefly, U937 cells were treated for various timepoints with or without chemicals. One hour before the harvesting, 2 μ l of a 1 mM solution of the biotinylated caspase-3 inhibitor Z-VDK-fmk (provided in the kit) were added to obtain a final concentration of 10 μ M in culture medium and the culture maintained at 37°C. Z-VDK-fmk forms a stable thioether bond with cysteine on the active site of caspase-3. Cells were then harvested, washed twice by centrifugation with cold PBS and lysed by addition of extraction buffer (1X) provided by the manufacturer. The concentration of human active caspase-3 in cell lysates was then determined by ELISA according to the manufacturer instructions.

Inhibition of Caspase-Dependent Apoptosis

U937 cells were treated or not with DNCB (3 μ g/ml) in a 96-well plate incubated with 10 or 50 μ M of the pan-caspase inhibitor Z-VAD-fmk. After a 48 hr culture period, cells were harvested and stained with Annexin V-FITC and PI for the evaluation of apoptosis inhibition using flow cytometry. Cells were stained separately for cell surface CD86 expression and Annexin V measurement.

Statistical Analysis

The Student's *t*-test was used to analyze data for significant differences. Values were regarded as significant at $p < 0.05$.

RESULTS

CD86 is Up-Regulated at Moderately Cytotoxic Concentrations of DNCB and NiSO₄

Dose-response studies of CD86 expression and cell viability were simultaneously performed on U937 cells following incubation for 48 hr with different contact sensitizers. Cell viability was assessed using flow cytometry with propidium iodide (PI) staining. Evaluation of the level of CD86 positive cells was restricted to gated viable cells (PI negative). Results were expressed as "stimulation index" calculated as follows: CD86-S.I. = [(% positive cells in the assay)/(% positive cells in the control)] $\times$ 100.

As shown in Figure 1A, the three contact sensitizers DNCB, NiSO₄, and pPD up-regulated CD86 expression dose-dependently. This effect was more pronounced with DNCB and NiSO₄, with CD86-S.I. greater than 800 and 350, respectively, at the highest tested concentration (3 μ g/ml and 50 μ g/ml, respectively). In parallel, DNCB and NiSO₄, when tested at the highest concentration, impacted cell viability, reaching levels below 70% (Figure 1B). In contrast, in the presence of pPD,

CD86-S.I. did not exceed 200 to 250 and cell viability was not affected.

Two scenarios emerged from this dual observation. In the first one, CD86 up-regulation was observed whereas cell viability was not to slightly modified compared to control cultures. This was the case for DNCB tested at 2 μ g/ml and pPD. In the second one, both parameters were simultaneously affected as illustrated by DNCB and NiSO₄ tested at 3 and 50 μ g/ml, respectively.

Attention was focused on the second case, when CD86 up-regulation was accompanied by moderate cytotoxicity. Questions about the origin of this cytotoxicity were addressed, as well as its potential involvement in allergen non-specific CD86 increased expression.

DNCB and NiSO₄ Induce Apoptosis in U937 Cells

To elucidate the mechanisms of cytotoxicity induced by test chemicals, kinetic studies of apoptosis and necrosis were conducted on the U937 cell line. Briefly, cells were treated at different time-points with given concentrations of chemicals, previously determined as moderately cytotoxic (25 to 35%) at 48 hr. For apoptosis evaluation, surface phosphatidylserine externalization on the cell membrane was quantified using Annexin V staining and flow cytometry (Martin et al., 1995). Evaluation of the respective populations was performed as detailed in the Materials and Methods.

In nontreated cells, apoptosis was always around 2 to 3% whereas in the presence of DNCB, 13% of apoptotic cells were detectable as early as at 7 hr (Figure 2). This level doubled at 24 hr and reached a maximum of $26 \pm 9\%$ at 48 hr. Secondary necrosis was present at 24 hr (17%) and 48 hr ($34 \pm 16\%$). NiSO₄ also induced a significant increase in apoptotic cells at 16 hr, with a steady increase of the apoptotic population from 14% at 16 hr up to $33 \pm 9\%$ at 48 hr. In parallel, secondary necrosis was detectable at 16 hr to reach $25 \pm 10\%$ at 48 hr.

All these results indicate that the two contact sensitizers, DNCB and NiSO₄ induced apoptosis of U937 cells, an event that preceded the appearance of dead cells.

The Cytotoxicity Observed at 48 hr is the Result of Hapten-Induced Apoptosis

To clarify the dynamics and relationship between apoptotic and necrotic events, the ratio (A/N) of Annexin V⁺/PI⁻ apoptotic cells over PI⁺ necrotic cells was calculated at 7, 16, 24, and 48 hr timepoints. We further investigated the dynamics of cell death to better understand the cytotoxicity observed at 48 hr. Figures 1 and 3 summarize results obtained with chemicals representative of different profiles obtained in U937 cells. On the one hand, two kinds of response were generated by contact sensitizers: DNCB and NiSO₄ induced a type A response involving both CD86 up-regulation and moderate cytotoxicity (25 to 35%), whereas the oxidative dye pPD, at least at tested concentrations, induced a type B response showing up-regulation of CD86 expression in the absence of a significant cytotoxicity.

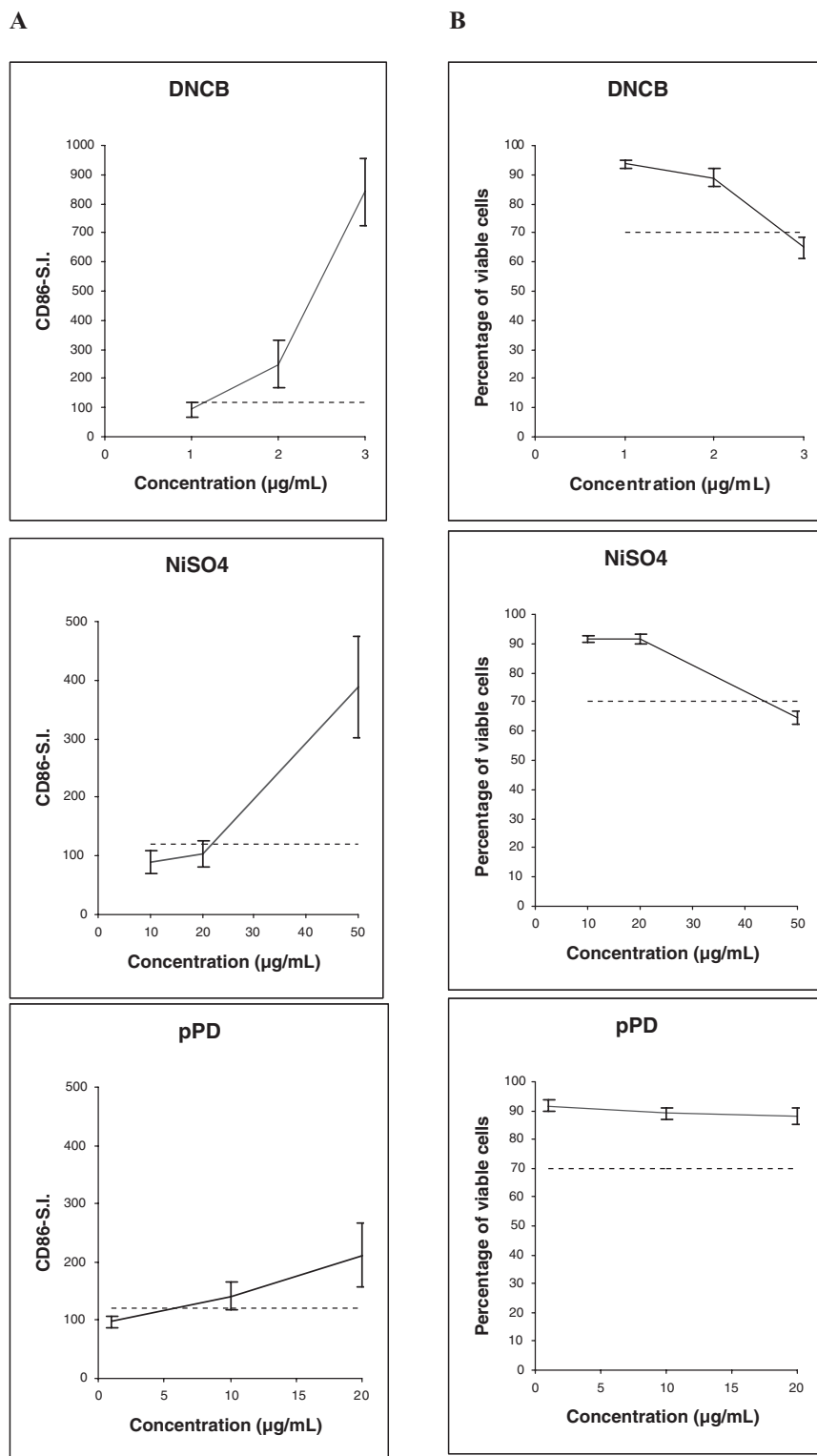


FIG. 1. Effect of contact sensitizers on CD86 expression and cell viability in U937 cells. (A) Expression of CD86 in U937 cells treated with DNCB, NiSO₄, or pPD. U937 cells were treated with various concentrations of test chemical for 48 hr and examined for the surface expression of CD86 using flow cytometry. Data on CD86 expression are presented as the ratio of the percentage of CD86-positive cells after treatment to the percentage of CD86-positive cells without treatment multiplied by 100: the ratio is termed stimulation index (S.I.). Data are representative of three independent experiments. (B) Cell viability of U937 cells treated with DNCB, NiSO₄, or pPD. U937 cells were treated with various concentrations of DNCB, NiSO₄, or pPD for 48 hr and examined for cell viability using nuclear staining with propidium iodide. Data are representative of three independent experiments.

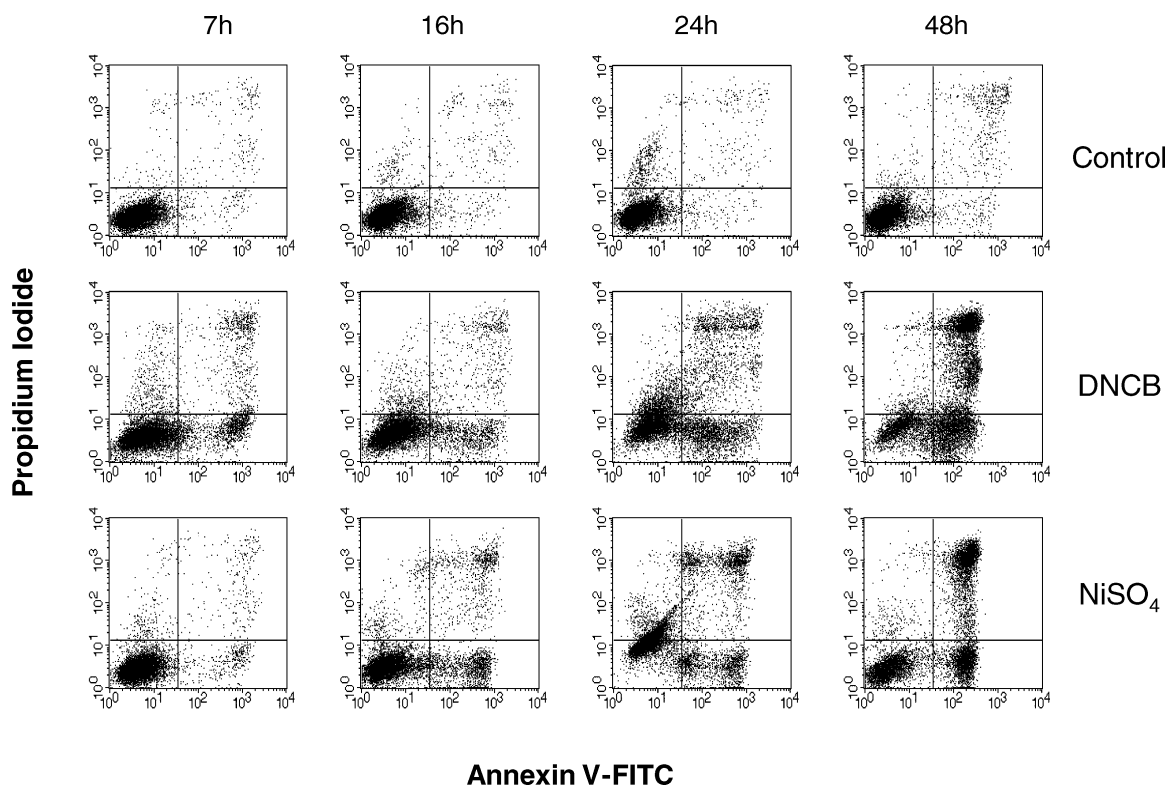


FIG. 2. Measurement of apoptosis of U937 cells treated with DNCB (3 $\mu\text{g/ml}$) or NiSO₄ (50 $\mu\text{g/ml}$). U937 cells were examined for surface expression of Annexin V (x-axis) and nuclear staining with PI using flow cytometry (y-axis) as described in Materials and Methods at 7, 16, 24, and 48 hr after treatment with the chemical. Results shown are from one representative experiment out of three.

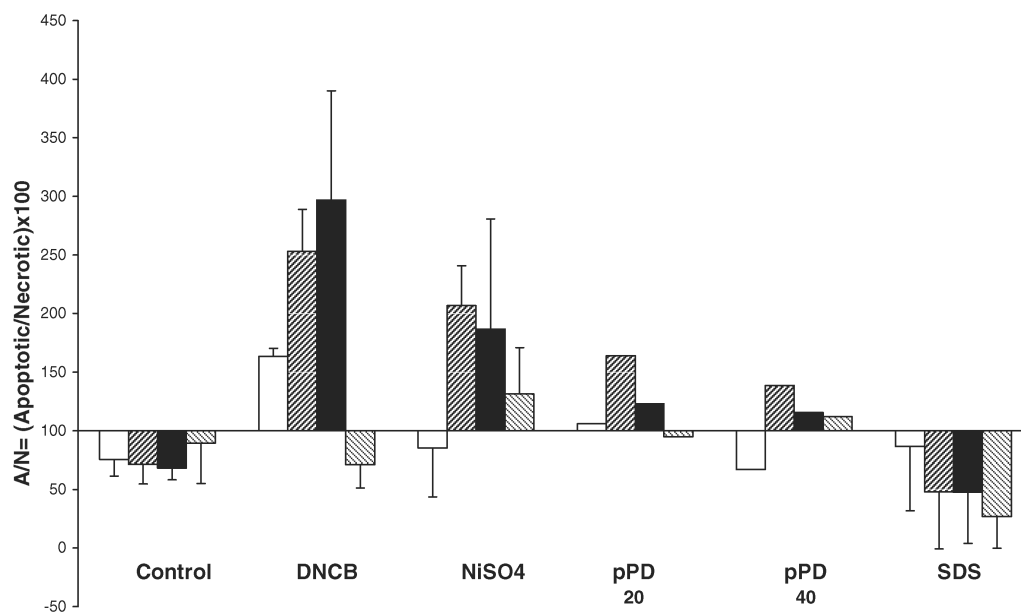


FIG. 3. Dynamics of cell death in U937 cells treated with test chemicals. U937 cells were treated with DNCB (3 $\mu\text{g/ml}$), NiSO₄ (50 $\mu\text{g/ml}$), pPD (20 40 $\mu\text{g/ml}$) or SDS (100 $\mu\text{g/ml}$) for 7, 16, 24, and 48 hr and examined for surface expression of Annexin V and nuclear staining with PI. Results are expressed as a ratio of Annexin V⁺PI⁻ apoptotic cells over PI⁺ necrotic cells from three independent experiments. For practical reasons, i.e., interference of cell dye with the fluorescence detection in the presence of pPD, results are representative of one experiment for each concentration. □: 7 hr; ▨: 16 hr; ■: 24 hr; ▩: 48 hr.

On the other hand, the irritant SDS was representative of a type C response with no reproducible effect on CD86 expression and enhanced cytotoxicity.

Timepoint analysis (Figure 3) revealed a common effect of the three contact sensitizers. Unlike the control and DMSO treated cells whose A/N ratio was nearly between 70 and 90, cells exposed to contact sensitizers showed a A/N ratio constantly higher than 100 during the first 24 hr and becoming lower than 100 during the following 24 hr. Taken together, these results suggested that contact sensitizers directly induced apoptosis and then cytotoxicity observed at 48 hr seems to be an indirect effect, such as necrosis. The result was the same whether U937 response was type A or type B. On the opposite, non-sensitizer SDS, which is representative of type C response, directly induced necrosis of U937 cells as demonstrated by A/N ratio below 50% as early as at 16 hr.

Altogether, a 48-hr treatment of U937 cells with the different test chemicals resulted in cytotoxic effects. In the case of contact sensitizers, cytotoxicity resulted from apoptosis whereas in the case of the irritant SDS, cytotoxicity resulted from direct necrosis.

Implication of Caspase-3 in the Apoptotic Process Induced by DNCB and NiSO₄

As the cysteine protease caspase-3 is a key executioner of apoptosis, responsible for the proteolytic cleavage of major proteins, its potential implication in the apoptotic process induced by DNCB and NiSO₄ was investigated. A kinetic study was performed on U937 cells treated or not with test chemicals. Active caspase-3 was quantified using an immunoassay. As shown in Figure 4, the level of active caspase-3 was increased in the presence of NiSO₄ or DNCB at 16 and 24 hr. However, after a 48-hr treatment with NiSO₄, the level of active caspase-3 was similar to that of untreated cells, whereas it was still increasing in DNCB-treated cells.

Taken together, these results suggested the implication of caspase-3 in the apoptotic process induced by DNCB and NiSO₄ in U937 cells. They also indicated that, different kinetics of caspase-3 activation were induced depending on the chemical.

CD86 Expression and Apoptosis Induced by DNCB in U937 Cells are Independent Events

Concomitant increase in CD86 expression and apoptosis in U937 cells stimulated by moderated cytotoxic doses of chemical allergens suggested a possible interdependence of the two events. Experiments were then designed to evaluate the impact of apoptosis inhibition on CD86 expression. DNCB, previously shown to induce both effects, was used as a prototype. U937 cells were simultaneously treated with DNCB (3 μ g/ml) and various concentrations of Z-VAD-fmk, the general caspase inhibitor (Zhu et al., 1995). Maximal noncytotoxic dose for Z-VAD-fmk was previously determined in untreated U937 cells and set at 50 μ M (data not shown). After a 48-hr culture pe-

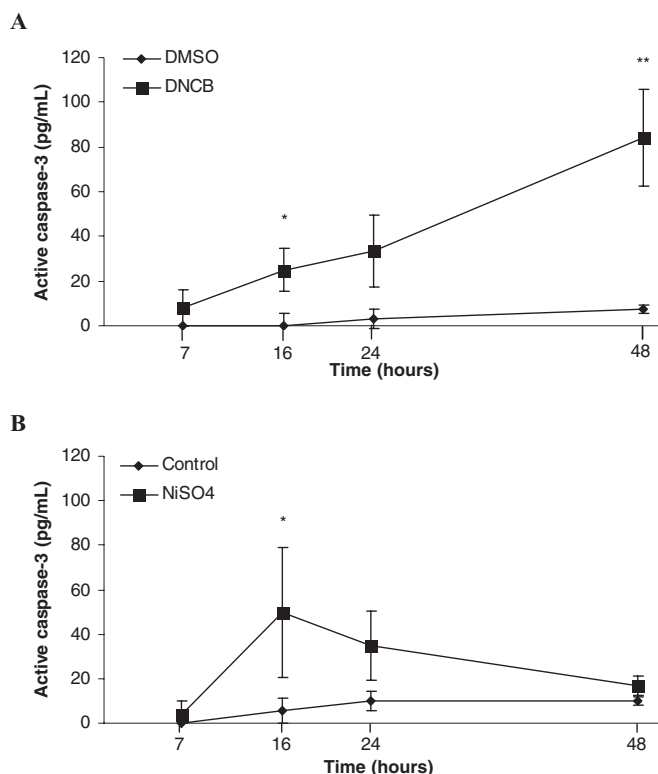


FIG. 4. Quantification of active caspase-3 in U937 cells treated with DNCB or NiSO₄. U937 cells were treated with DNCB (3 μ g/ml) or NiSO₄ (50 μ g/ml) for different time periods. One hr before harvesting, biotinylated Z-VAD-fmk was added. Cell extracts were measured for active caspase-3 by ELISA. * $p < 0.05$, ** $p < 0.01$.

riod, apoptosis (Annexin V staining) and CD86 expression were assessed using flow cytometry. A representative experiment is reported on Figure 5. In the presence of 50 μ M of Z-VAD-fmk, apoptosis was inhibited by 54% whereas nearly no effect was observed at 10 μ M (Figure 5A). Statistical analysis performed on 3 independent experiments indicated that apoptosis decrease was significant ($p < 0.05$) whereas CD86 expression level was not significantly different ($p > 0.05$). CD86 expression, however, showed a constant 20% inhibition irrespective of Z-VAD-fmk level that strongly suggests a rather nonspecific effect of Z-VAD-fmk effect (Figure 5B).

These results indicate that caspases seem to play a pivotal role in DNCB-mediated apoptosis, but only a minor role, if any, in CD86 up-regulation. Moreover, these results demonstrate that the number of apoptotic U937 cells present in the culture does not affect the level of CD86 expression. Altogether, these results suggest that contact sensitizer-induced apoptosis and CD86 up-regulation is two independent events.

Apoptosis Does Not Interfere with the Quantification of CD86 Expression

Finally, it was essential to determine whether the presence of apoptotic cells could interfere or not with the

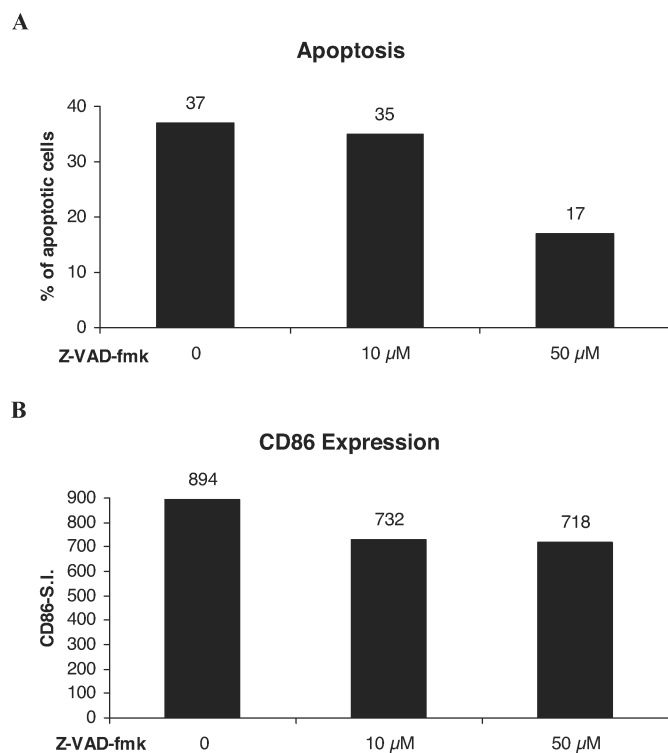


FIG. 5. Effect of apoptosis on chemical-induced CD86 expression. (A) Percentage of Annexin V⁺/PI⁺ apoptotic cells induced by chemical treatment with or without Z-VAD-fmk. U937 cells were treated for 48 hr with DNCB (3 μ g/ml) in the presence or in the absence of Z-VAD-fmk and examined for Annexin V- and PI-staining. (B) CD86 expression on U937 cells after treatment with chemicals and Z-VAD-fmk. Cells were treated with DNCB (3 μ g/ml) in the presence or in the absence of Z-VAD-fmk. Forty-eight hr after stimulation, the expression of CD86 was measured using flow cytometry. Results shown are from one representative experiment.

quantification of CD86 expression induced by contact sensitizers. DNCB- and NiSO₄-treated U937 cells were analyzed at 48 hr for CD86 expression and their apoptotic/necrotic status by CD86-PE/Annexin V-FITC/7-AAD triple staining. DNCB was tested at 2 μ g/ml as described for screening test (Rousset et al., 2002), with cell viability higher than 70% (see Figure 1B). As usual, flow cytometry analyses were systematically performed on 7-AAD negative cells, to exclude dead cells from the analysis. Then, the percentage of CD86 expressing cells was alternatively assessed on the whole gated population (7-AAD negative, Annexin V positive and negative cells) corresponding to a mixed population of apoptotic and nonapoptotic cells, or on the cell population restricted to the viable, non-apoptotic cells (7-AAD negative, Annexin V negative cells). As reported in Table 1, both methods gave similar CD86-S.I. values (statistical analysis shows no significant difference, $p > 0.7$) demonstrating that the measurement of CD86 expression induced by DNCB and NiSO₄ in U937 cells was not affected by the presence of apoptotic cells within the analysis gate. Moreover, analysis of dot-plots revealed that the majority of CD86 expressing cells were not apoptotic (data not shown).

TABLE 1

Stimulation indexes of CD86 expression in non-dead U937 cells treated with DNCB or NiSO₄

	CD86-S.I. on 7-AAD ⁻ cells	
	Annexin V ⁻ and Annexin V ⁺	Only Annexin V ⁻
Control	100	100
DNCB	365 $\pm$ 67	325 $\pm$ 148
NiSO ₄	395 $\pm$ 111	392 $\pm$ 42

Cells have been treated with DNCB (2 μ g/ml) or NiSO₄ (50 μ g/ml) for 48 hr. Flow cytometry analysis of CD86 expression and Annexin V labeling was systematically performed on 7-AAD negative cells. Apoptotic cells (Annexin V⁺) were alternatively included (left column) or excluded (right column) from the analysis. Results are mean $\pm$ SD of 3 independent experiments.

These results confirmed that apoptosis is not a confusing factor for the establishment of a "CD86 stimulation index."

DISCUSSION

CD86 up-regulation is a hallmark of the effect of contact sensitizers on DC and myeloid cell lines (Aiba et al., 1997; Rousset et al., 2000, 2002; Ashikaga et al., 2002; Boisleve et al., 2005; De Smedt et al., 2005; Azam et al., 2006). However, several authors addressed the question of the specificity of this marker since depending of the allergens a significant increase in CD86 expression was concomitant to moderate cytotoxicity (Hulet et al., 2005; Straube et al., 2005). Using a MoDC model, Hulet et al. reported that non-sensitizer chemicals, even though they induced moderate cytotoxicity, did not significantly potentiate CD86 expression. In order to setup robust alternative tests, their final recommendation was to treat DC with concentrations of contact sensitizers that induced slight to moderate cytotoxicity (Hulet et al., 2005).

The aim of this study was to evaluate the possible implication of cytotoxicity in CD86 detection. For this purpose, experiments were conducted on the U937 myelomonocytic cell line that we and others have reported as a good candidate for developing an *in vitro* test method to predict contact sensitivity with the advantage of relying on unlimited and reproducible material (Rousset et al., 2002; Python et al., submitted for publication). The nature of cell death (apoptosis/necrosis) was also investigated.

The observations reported here showed that the contact sensitizers DNCB, NiSO₄, and pPD directly induced CD86 expression and apoptosis, whereas the irritant SDS did not produce such effects but rather directly induced necrosis. Kinetic studies revealed that apoptosis was detectable at early timepoints and took place at least 8 hr before cytotoxicity (PI⁺ dead cells) was noticed. Dynamics of the apoptosis and necrotic events was clearly apparent when apoptosis/necrosis ratio was calculated and showed an inversion of the ratio at 48 hr with more dead cells than apoptotic cells at this latest timepoint. Moreover,

when apoptosis was significantly inhibited ($p < 0.05$) by the pan-caspase inhibitor Z-VAD-fmk, the CD86-S.I. was only marginally affected. These data are in agreement with the fact that CD86 up-regulation can be obtained in the absence of significant cytotoxicity as shown for DNCB at 2 $\mu\text{g}/\text{ml}$ and pPD. Finally, triple staining experiments performed with DNCB treated cells, demonstrated that CD86 up-regulation preferentially concerned non apoptotic cells (Annexin V⁻/7-AAD⁻). This indicated that the presence of apoptotic cells in the assay did not change the ranking of the CD86-S.I. and, more importantly, that the presence of cells engaged in death process at the time of CD86 testing did not interfere with the test by itself.

As previously described on MoDC (Manome et al., 1999), this study also pointed out the occurrence of hapten-induced apoptosis on the myelomonocytic U937 cell line. This event was observed at early times after chemical or metal allergen addition and leads to cell death (25 to 35%) at the time where CD86 detection was performed. Even if more marked when investigated at moderately toxic concentrations, results obtained with pPD indicated that apoptosis, as well as an increase in CD86 expression could be induced at non toxic concentrations. Several molecules have been reported to induce apoptosis in U937 cell line (Fujita and Tsuruo, 1998; Wright et al., 1998). Among these molecules, some such as lidocaine (Kamiya et al., 2005) and octylcaffate (Ujibe et al., 2005) are contact allergens. Careful analysis of results generated on MoDC (Manome et al., 1999) and U937 cells strongly suggests that apoptosis is specifically related to contact sensitizers, whereas non sensitizers produce direct cytotoxicity (personal preliminary data).

In the case of DNCB, apoptosis was at least partly caspase dependent since it was inhibited by Z-VAD-fmk at a maximal non cytotoxic dose (50 μM). Moreover, caspase-3, a key executor of apoptosis leading to DNA fragmentation and chromatin condensation (Enari et al., 1998; Sakahira et al., 1998), was activated in response to both DNCB and NiSO₄. Kinetic studies of caspase-3 activation revealed that DNCB activated caspase-3 time-dependently whereas activation by NiSO₄ was transient. Kim et al. (2002) reported similar results with a T-cell hybridoma model where nickel induced apoptosis with activation of caspase-3 peaking at 18 hr. Apoptosis in response to metal haptens could be a common feature since nickel and cobalt were described to induce apoptosis in MoDC (Granchi et al., 1998; Manome et al., 1999), as well as in the U937 cell line. The situation with the organic allergen DNCB was not as clear. Whilst DNCB constantly induced strong apoptosis in U937 cells throughout the culture period (48 hr), it was classified as non apoptosis-inducer by Manome et al. (1999) when tested in MoDC at two fixed timepoints (6 and 12 hr). Possible explanations for this discrepancy are: (i) difference between the two cellular models, where U937 are proliferating cells and MoDC are not proliferating cells submitted to donor to donor variations and, (ii) the tested concentrations of DNCB are not comparable (15 μM on U937 cells versus 900 μM on MoDC), concentrations applied to MoDC being highly cytotoxic in the U937 model, even

at the early timepoints 6 and 12 hr. Comparison performed on more detailed dose-response and kinetic studies in the two cellular models would definitively permit to evaluate to what extent MoDC and U937 cellular models are similar or divergent.

Taken together, our data demonstrated that at slight to moderate cytotoxic concentrations, the contact sensitizers NiSO₄, DNCB, and pPD induced CD86 up-regulation and apoptosis in U937 cells. This suggests that a combination of two different and independent cellular markers could be used to predict contact sensitivity *in vitro* using a myelomonocytic cell line such as U937. Since we demonstrated that at the time of CD86 determination, cytotoxicity arising from early apoptosis did not interfere with the ranking and the specificity of CD86 marker, and in agreement with Hulette et al. (2005), we propose to set up a predictive test method based on the use of a panel of not to moderate cytotoxic chemical doses with a limit of viability set at 70%.

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