The Sensitizer 2,4-Dinitrofluorobenzene Activates Caspase-3 and Induces Cell Death in a Skin Dendritic Cell Line

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In this work, a dendritic cell line derived from mouse skin (FSDC) was used, as an in vitro experimental model, to evaluate the cytotoxic effect of two chemical sensitizers, a strong sensitizer (2,4-dinitrofluorobenzene, DNFB) and a weak sensitizer (2,4-dichloronitrobenzene, DCNB). The results indicated that DNFB reduces the cellular metabolism of FSDC, as evaluated by the reduction of the tetrazolium salt, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). All the DNFB concentrations tested, ranging from 5.2 μM to 26 μM, significantly inhibited the MTT reduction after 1 hour of cell exposure to the sensitizer. In contrast, incubation of FSDC with the weak sensitizer DCNB had no significant effect on the MTT reduction assay. When the cells were incubated with DNFB (13 μM), for 3 and 6 hours, morphological changes characteristics of cell death by apoptosis were observed, as assessed by propidium iodide (PI) DNA staining and annexin-V externalization analysis. These results correlate well with an increase of caspase-3–like activity after FSDC exposure to DNFB (13 μM) for 6 hours. Together, these results indicate that apoptotic death of skin dendritic cells occurs after exposure to the sensitizer DNFB, although necrotic cell death was also observed when the cells were incubated with high concentrations of DNFB (26 μM), or after long periods of cell exposure to the chemical DNFB (13 μM, for 6 hours).

Skin dendritic cells (DCs) are an epidermal population of cells characterized by their potent antigen-presenting capacity (Bancherau and Steinman 1998). They capture the antigen and leave the epidermal environment, migrating to the lymph nodes, where they contact with and activate naive T cells (Enk 1997). The process of DC migration is a tightly regulated event, dependent on allergens and epidermal cytokines, which induce the expression of cell surface markers essential for antigen presentation, such as the major histocompatibility complex class II molecules and costimulatory molecules (Bancherau and Steinman 1998).

Recent studies demonstrated that DCs are present in afferent lymph, but not in efferent lymph, suggesting that most of the migrating DCs die after their arrival to the lymph nodes (Banchereau and Steinman 1998). It was also reported that in tissue culture, fully differentiated or mature DCs undergo rapid apoptosis (Ludewig et al. 1995; Josien et al. 2000), unless they receive a survival signal from T cells, which occurs after interaction of T cells with activated DCs (De Smedt et al. 1998). In addition, other recent evidences suggest that once DCs have interacted with T cells, they complete their differentiation process and die by apoptosis (Winzler et al. 1997; Matsue et al. 1999).

The aim of this work was to evaluate the effect of standard sensitizers on the metabolism and on the viability of a purified population of skin DCs. A dendritic cell line derived from fetal mouse skin (FSDC) was used as an in vitro experimental model exhibiting phenotypic and functional characteristics of immature DCs (Girolomoni et al. 1995). The effect of a strong contact sensitizer (2,4-dinitrofluorobenzene, DNFB) (Reiser and Schneeberger 1996) and a weak sensitizer (2,4-dichloronitrobenzene, DCNB) (Basketter et al. 1996) was tested on the metabolism of FSDC, as determined by the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

(MTT), and on the decision of the cells to die by apoptosis or necrosis, as determined by propidium iodide (PI) DNA staining and annexin-V expression on cell surface (Rescigno et al. 2000). Since caspases, a group of intracellular cysteine proteases, participate in the initiation and execution phases of apoptotic cell death (Fadeel, Orrenius, and Zhivotovsky 2000), we studied the ability of DNFB to activate caspase-3.

MATERIALS AND METHODS

Materials

DNFB and DCNB were purchased from Sigma-Aldrich Química (Madrid, Spain). Fetal calf serum was obtained from Biochrom KG (Berlin, Germany) and trypsin was from Gibco (Paisley, UK). The protease inhibitor cocktail was purchased from Roche (Carnaxide, Portugal). Propidium iodide was obtained from Molecular Probes Europe (Leiden, The Netherlands). The caspase-3–like enzyme substrate, Ac-asp-glu-val-asp-pNA (DEVD-pNA), was from Bachem (Bubendorf, Switzerland). All other reagents were either from Sigma Chemical Co. (St. Louis, MO, USA) or from Merck (Darmstadt, Germany).

Cell Culture

FSDC was kindly provided by Dr. G. Girolomoni (Girolomoni et al. 1995). The cells were cultured in Iscove's medium (Sigma) supplemented with 10% fetal calf serum, 1% glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin.

MTT Assay

Assessment of MTT reduction by metabolically active cells was carried out using a colorimetric assay (Mosman 1983). In this method, the optical density of the solution containing the formazan produced by metabolically active cells is measured spectrophotometrically. The cells (0.2×10^6 cells/well, cultured for 24 hours in 48-well microplates) were incubated for 1, 3, 6, and 12 hours with varying concentrations of two different chemical compounds, DNFB (5.2, 13, and 26 μM) or DCNB (5.2, 13, and 26 μM), or the corresponding vehicle, dimethyl sulfoxide. After removal of cell-free supernatants, 400 μl of culture medium and 40 μl of MTT solution (5 mg/ml in phosphate-buffered saline [PBS]) were added to each well. The microplates were further incubated at 37°C for 1 hour, in a humidified atmosphere of 95% air/5% CO_2. Supernatants were then discarded and 300 μl of acidified isopropanol (0.04 N HCl in isopropanol) were added to the cultures and mixed thoroughly to dissolve the dark blue crystals of formazan. Formazan quantification was performed using an automatic plate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm. The dimethyl sulfoxide concentration in the culture medium never exceeded 0.1%, and the assay of cellular MTT reduction in the presence of this concentration of dimethyl sulfoxide revealed no toxic effect (data not shown).

Detection of Apoptosis With Annexin-V Staining

Cells grown on coverslips (0.2×10^6 cells/well, cultured during 24 hours in 48-well microplates) were incubated for 1, 3, or 6 hours with varying concentrations of DNFB (5.2 to 26 μM) in a final volume of 400 μl/well. After removal of cell-free supernatants, the cells were washed twice with sodium medium (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4). They were subsequently stained, in the dark, for 5 minutes, with PI (1 μg/ml) and fluorescein isothiocyanate–labeled annexin-V (1 μg/ml), dissolved in sodium medium. Fluorescence images were obtained using 488 nm (excitation) and 568 nm (emission) line of a krypton/argon mixed gas laser, on a Bio-Rad MRC 600 fluorescent confocal microscope.

Assaying of Caspase-3–Like Activity

For caspase-3–like enzymatic activity analysis, the cells (2×10^6 cells/well, cultured during 24 hours in 6-well microplates) were treated with culture medium (control), or with DNFB (5.2 to 26 μM), for 1, 3, and 6 hours. After treatment, the cells were washed twice with KPM (50 mM KCl, 50 mM PIPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.0) and further washed once with KPM supplemented with 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, and the protease inhibitor cocktail (complete KPM). Next, the cells were lysed with complete KPM containing 0.5% (v/v) Triton X-100, and crude extracts were prepared by two cycles of repeated freezing and thawing, followed by centrifugation at $18000 \times g$, for 10 minutes at 4°C. Protein concentration in the supernatant was determined using the bicinchoninic acid solution (Sigma). Cells lysates (30 μg of protein) were incubated with 100 μl of the reaction buffer composed of 25 mM Hepes, pH 7.5, 0.1% CHAPS (w/v), 10 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, and the protease inhibitor cocktail. The reaction was started by the addition of 100 μM DEVD-pNA, followed by incubation at 37°C for 2 hours. Caspase-3–like activity was determined by monitoring the release of the cleaved substrate, every 15 minutes, using an automatic plate reader (SLT), at 405 nm. The results are expressed as a percentage of the control rate of substrate cleavage.

Data Analysis

Results are presented as mean $\pm$ *SEM* of the indicated number of independent experiments. Mean values were compared using one-way analysis of variance (ANOVA) and the Bonferroni's multiple comparison test. The significance level was .05.

RESULTS

The Contact Sensitizer DNFB Reduces the Metabolism of FSDC Cells

As shown in Figure 1*A*, the strong contact sensitizer DNFB decreased the reduction of MTT by FSDC, with a rapid onset.
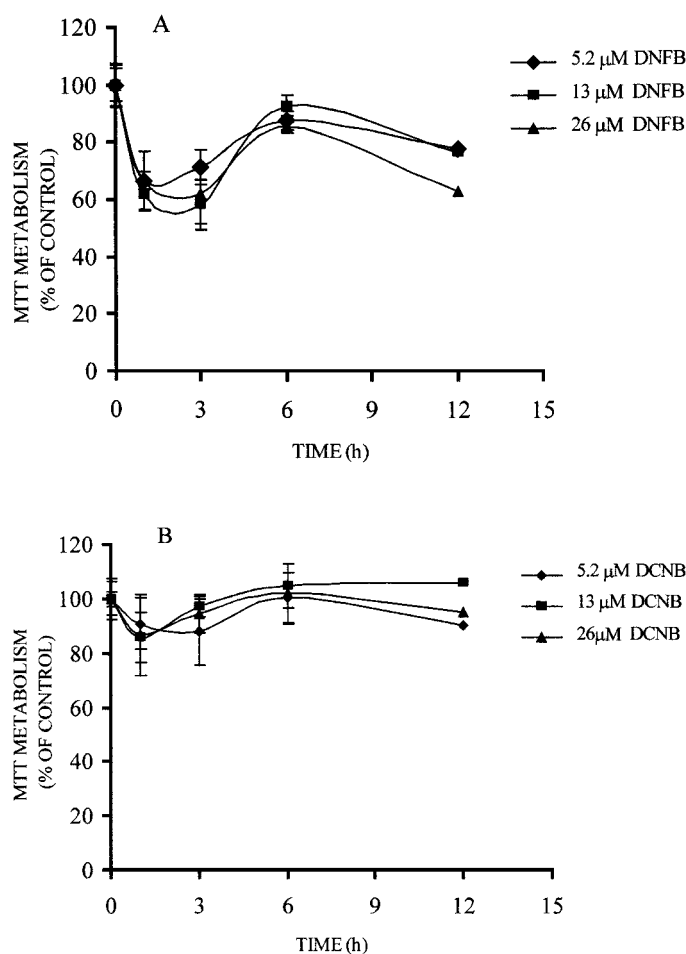
FIGURE 1

Effect of the exposure period to the chemicals DCNB and DNFB on the FSDC metabolism (MTT assay). The FSDC cells were exposed to different concentrations of DNFB (*A*) or DCNB (*B*), for 1, 3, 6, and 12 hours. The MTT assay was performed as described in Materials and Methods. Results are expressed as a percentage of MTT metabolism by control cells maintained in culture medium. Each value represents the mean $\pm$ *SEM* from five experiments, performed in duplicate.

In contrast, incubation of FSDC with DCNB, at concentrations ranging from 5.2 to 26 μM, for as long as 12 hours, had no significant effect on MTT reduction (Figure 1*B*). All concentrations of DNFB tested (5.2, 13, and 26 μM) inhibited MTT reduction by about 33% to 38% within the first hour of exposure (Figure 1*A*). A recovery period in MTT reduction was observed when the activity was measured in cells exposed to DNFB for 6 hours, but continuous exposure of FSDC to this sensitizer for 12 hours further evoked an inhibition of MTT reduction (Figure 1*A*). In the latter experimental conditions, and in comparison to the control value, the amount of blue formazan precipitates formed decreased to 77.3% $\pm$ 3.2% ($p < .001$), 76.4% $\pm$ 3.7% ($p < .001$), and 63.0% $\pm$ 1.8% ($p < .001$) when the cells were incubated with 5.2, 13, or 26 μM DNFB, respectively (Figure 1*A*).

The Contact Sensitizer DNFB Induces Apoptotic Death of FSDC Cells

We next examined the mode of cell death in FSDC incubated with DNFB. Cell death was measure with PI, a membrane impermeant dye that stains red the DNA of cells with a disrupted plasma membrane, and fluorescein isothiocyanate–labeled annexin-V, which binds to the phosphatidylserine residues translocated to the external leaflet of the plasma membrane in cells undergoing apoptosis (Fadok et al. 1998; Rescigno et al. 2000). Viable cells with intact plasma membrane are therefore not stained with PI or with annexin-V. Apoptotic cells are not stained with PI, but because there is phosphatidylserine translocation to the external leaflet of the plasma membrane, they bind annexin-V. Necrotic cells are permeable to PI, which stains DNA, and are also permeable to annexin-V, which stains phosphatidylserine on the inner leaflet of the plasma membrane.

The results shown in Figure 2 were obtained by confocal microscopy and show that control FSDC cells do not display fluorescence after staining with fluorescein isothiocyanate–conjugated annexin-V and PI (Figure 2*A*), and that apoptosis does not occur after cell exposure to 13 μM DNFB for 1 hour (Figure 2*B*). However, after 3 to 6 hours of exposure to the sensitizer, cells in apoptotic stage were detected (Figure 2*C*, *D*). Incubation of the cells with the weak sensitizer compound DCNB, at 13 μM, for 3 hours, did not induce apoptosis (data not shown). When FSDC cells were exposed to higher concentrations of DNFB (26 μM), for 1 hour (data not shown), or when they were exposed to 13 μM DNFB, for 6 hours (Figure 2*D*), necrotic cells were also observed, with their nuclei labeled with PI, as indicated by arrows in Figure 2*D*. However, in Figure 2*D*, the large majority of the cells was stained by annexin-V, but not by PI, indicating that cell death occurred mostly by apoptosis.

DNFB Induces Caspase-3–Like Enzyme Activation in FSDC

Because caspase-3 plays a key role in the execution phase of apoptotic cell death (Fadeel, Orrenius, and Zhivotovsky 2000), we next investigated caspase-3–like enzyme activation induced by DNFB, in FSDC, using the specific peptide substrate DEVD-pNA. As shown in Figure 3, 6 hours exposure to 13 μM DNFB induced caspase-3–like enzyme activation, from 100% (control cells) to 173.23% $\pm$ 13.45%. However, DCNB or 1 and 3 hours exposure to DNFB did not induce caspase-3–like activity in FSDC (not shown).

DISCUSSION

The knowledge of the signaling events triggered in DCs by different sensitizers could help to modulate the skin
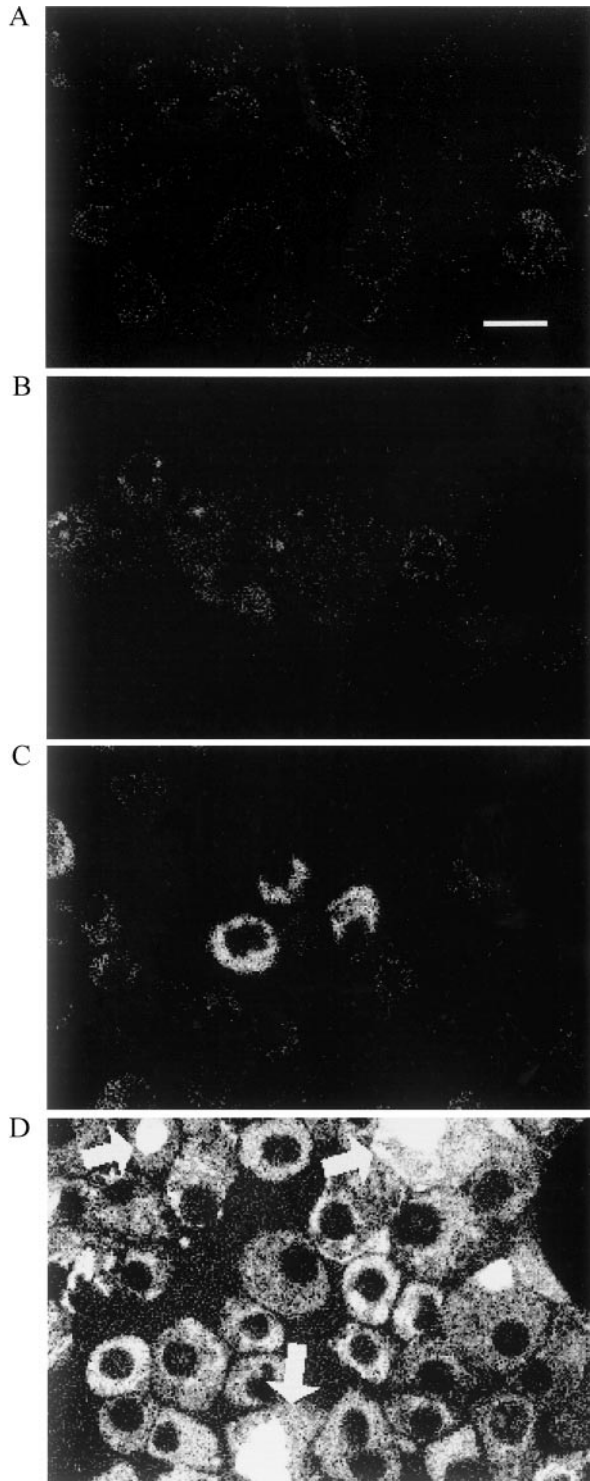
FIGURE 2
DNFB induces apoptosis in FSDC. FSDC cells were stained
with propidium iodide (1 μg/ml) and fluorescein
isothiocyanate–annexin-V (1 μg/ml), as described in Materials
and Methods, after incubation with 13 μM DNFB. (*A*) Control;
(*B*) after 1, (*C*) 3, or (*D*) 6 hours exposure to DNFB. The
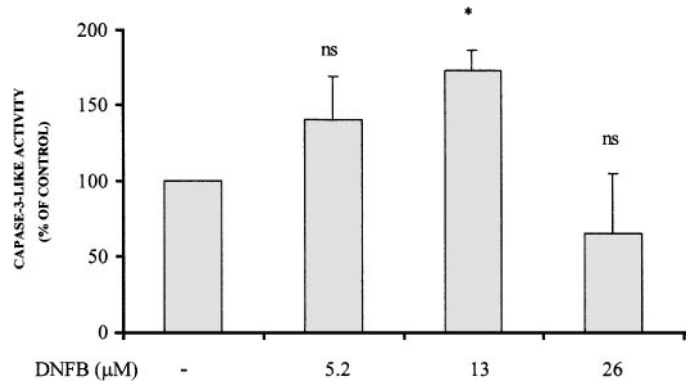arrows indicate necrotic cells. Scale bars = 20 μm.

FIGURE 3
DNFB induces apoptosis in FSDC by activating the caspase-3.
FSDC cells were treated with culture medium (control), or
were exposed to different concentrations of DNFB, for 6 hours.
Caspase-3–like activity was measured as described in
Materials and Methods. The results are expressed as a
percentage of the control rate of substrate (DEVD-pNA)
cleavage, measured at 405 nm, every 15 minutes. Each value
represents the mean $\pm$ *SEM* from five experiments, performed
in duplicate (*$p < .05$; ns, not significant).

inflammatory processes. In this work, the effects of two chemical sensitizers, a strong contact sensitizer (DNFB) and a weak sensitizer (DCNB), on the metabolism and on the viability of a DC line derived from fetal mouse skin (FSDC), which is representative of immature skin DC precursors (Girolomoni et al. 1995), were evaluated. The doses used in this work (5.2, 13, and 26 μM) are in agreement with those used by other authors who performed in vitro studies in DCs (e.g., Manome, Aiba, and Tagami 1999). Our results show that only the strong sensitizer, DNFB, decreases the FSDC metabolism (Figure 1), activates caspase-3–like enzymes, and induces apoptosis (Figures 2 and 3).

Incubation of FSDC with DNFB, at 13 μM, rapidly decreased MTT reduction within 1 hour (Figure 1*A*), but staining with annexin-V, which labels apoptotic cells, was detected only after 3 hours of exposure to the allergen (Figure 2*C*). Interestingly, the effect of DNFB on the metabolic activity (MTT assay) of FSDC showed a transient recovery phase after 6 hours exposure to the allergen (Figure 1*A*). This transient recovery of the MTT reduction during long-time exposure to DNFB suggests that this allergen causes a burst in the metabolic activity of the cells, which may result from DC activation by the sensitizer. In fact, DCs respond to sensitizing chemicals by producing cytokines and expressing costimulatory molecules (Aiba et al. 1997), and the level of phosphotyrosine increased in human DCs under stimulation with contact sensitizers (Kühn et al. 1998). The transient secondary increase of MTT reduction observed when the cells were continuously incubated with DNFB, for 6 hours, was followed by another phase of reduction of metabolic activity (Figure 1*A*), which is likely to correspond to cell death, mainly

by apoptosis. This may occur due to incapacity of the activated FSDC to present the antigen to a naive T cell in our experimental conditions. Accordingly, in monocytes-derived DCs, others sensitizers, such as $NiCl_2$ and $CoCl_2$, also induced apoptosis (Manome, Aiba, and Tagami 1999). In fact, in vivo, when mature DCs carrying the antigen are not stimulated through the CD40 molecule of the lymphocytes, they die by apoptosis (Thomas and Lipsky 1996).

Our results also show that DNFB caused death of a subpopulation of FSDC by necrosis. Necrotic FSDC cells were observed upon long periods incubation (>6 hours) with 13 μM DNFB (Figure 2D), or in the presence of higher concentrations of the allergen (data not shown). High concentrations of DNFB may cause energy failure and concomitant ATP depletion, precluding the activation of execution subroutines required for apoptotic cell death, leading to necrosis (Nicotera and Leist 1997). In contrast with the effects of DNFB, DCNB did not affect the ability of FSDC to reduce the MTT salt (Figure 1B) and was also without effect on annexin-V and PI staining (data not shown).

In contrast to necrotic cell death, apoptosis is a caspase-dependent process of cell death, with associated classical morphological features, such as chromatin condensation and fragmentation, cell shrinkage, formation of 'apoptotic bodies,' and phosphatidylserine externalization, which permits the labeling of cells for phagocytosis (Cohen 1997). In recent years, a family of cysteine proteases, termed caspases, have been implicated in the process of cell destruction by apoptosis (Fadeel et al. 2000). Caspase-3 is one of the key proteases of apoptosis, being responsible, at least in part, by the proteolytic cleavage of target proteins (Cohen 1997). Caspase-3 is widely distributed in mammalian cells, with high expression in cell lines of lymphocytic origin, suggesting that it may be an important mediator of apoptosis in the immune system (Cohen 1997). Here, we observed that 13 μM DNFB induced caspase-3–like activation in FSDC, after 6 hours exposure of the cells to the chemical (Figure 3). These results correlate with the effect of DNFB on the annexin-V binding to FSDC under the same experimental conditions (Figure 2D). In contrast, incubation of FSDC with 26 μM DNFB, for 6 hours, did not induce caspase-3–like activity (Figure 3), probably because the mode of cell death in this case was by necrosis.

Epidermal dendritic cells undergo apoptosis after ultraviolet B radiation (Takashima 1995; Kitajima et al. 1996), and recent studies suggest that cell death may facilitate antigen presentation and may be necessary for activation of the immune system (Chatergoon et al. 2000). Apoptotic cells (Rovere et al. 1998), and possibly necrotic cells (Sauter et al. 2000), were found to be sufficient to trigger DC maturation and the presentation of intracellular antigens from dying cells to lymphocytes.

In conclusion, our results suggest that DNFB induce cell death of dendritic cells, mainly by apoptosis. However, the mechanisms responsible for apoptotic death of dendritic cells upon exposure to the strong contact sensitizer and the consequences of this event in vivo remain to be determined.

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