Dexamethasone modifies the functional responses of the granulocytic differentiating HL-60 cells

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We have studied the effect of dexamethasone on the granulocytic differentiation of the human promyelocytic cell line HL-60 induced by treatment with retinoic acid (RA) or dimethyl sulphoxide (DMSO). Dexamethasone potentiated the immunophenotypic and functional parameters associated with the granulocytic differentiation induced by RA, including changes in CD11b and CD71 expression, inhibition of cell proliferation, enhancement of secretory and oxidative responses and increase of the phospholipase C (PLC), phospholipase A₂ (PLA₂) and phospholipase D (PLD) activities. However, dexamethasone had selective effects on several parameters of DMSO-induced cell differentiation. Dexamethasone inhibited the DMSO-induced increase of CD11b cell surface expression as well as the oxidative response and PLD activation triggered by 4 β -phorbol 12-

myristate 13-acetate. Nevertheless, dexamethasone potentiated the receptor-mediated PLC activation and the receptor-mediated secretory and oxidative responses in DMSO-treated cells. Unlike RA-treated HL-60 cells, the DMSO-treated cells contained high values of activatable PLA₂ activity which were not affected by dexamethasone. Thus dexamethasone affected differently functional parameters and effector systems of granulocytic HL-60 cells, depending on the differentiation agent used. Dexamethasone by itself did not induce HL-60 cell differentiation, but enhanced the receptor- and non-receptor-mediated secretory responses and induced the appearance of stimulated PLA₂ activity in undifferentiated HL-60 cells. These data provide evidence for the selective modulation of functional responses by dexamethasone through alterations in signalling processes.

INTRODUCTION

The human promyelocytic HL-60 cell line has been used as a model system to study granulocytic and macrophage differentiation [1]. These cells can be induced to differentiate towards granulocyte-like cells upon exposure to a variety of agents, such as dimethyl sulphoxide (DMSO) or retinoic acid (RA) [2]. Other compounds induce differentiation towards a monocyte/macrophage cell type, such as phorbol esters, 1 α ,25-dihydroxyvitamin D₃ or interferon- γ [2]. The molecular mechanisms involved in the differentiation process are largely unknown, but HL-60 cell differentiation has been associated with several morphological and functional changes, including loss of proliferative capacity, changes in mRNA levels of various nuclear proto-oncogenes, changes in expression of cell surface antigens, and intracellular alkalization [3–5]. Furthermore, the differentiating HL-60 cells acquire several functions of phagocytic cells, namely chemotaxis, cell adhesion, superoxide-anion production and lysosomal-enzyme release [6,7].

Glucocorticoids are potent immunosuppressive compounds. They affect several neutrophil functions, inhibiting chemotaxis, cell adhesion, superoxide-anion production and lysosomal-enzyme release stimulated by different agonists [8–10]. Glucocorticoids have been shown to act through cytoplasmic glucocorticoid receptors [11], one of their effects being the induction of inhibitors of intracellular phospholipase activities that interfere with cellular responses [12]. However, glucocorticoids promote other effects such as induction of expression of G-proteins or β -adrenergic receptors [13,14] that could modify signal-transduction processes [15,16]. Other studies suggest that glucocorticoids may also have a role in cell differentiation. Cortisol administration causes a dose-dependent increase in the number of granulocytic colonies

[17], and enhances the amount of circulating mature neutrophils by prolonging their half-life in circulation, releasing mature granulocytes from the bone marrow and decreasing the egress of neutrophils to sites of inflammation [18]. Previous studies have shown that dexamethasone inhibits differentiation of murine erythroleukaemia cells [19], and affects the expression of differentiation markers and the mRNA levels of different proto-oncogenes in the human promonocytic cell line U937 treated with DMSO or 4 β -phorbol 12-myristate 13-acetate (PMA) [20,21]. Furthermore, it has been recently reported that glucocorticoids interfere with functions of PMA-induced differentiating U937 cells, although they do not affect either the differentiation process or cell growth [22].

In the present work, we have investigated whether dexamethasone is able to induce HL-60 cell differentiation or to affect the DMSO- or RA-induced granulocytic differentiation of HL-60 cells. The cell differentiation process was followed through the expression of the cell-surface antigens CD11b, an adhesion molecule that functions as the C3bi receptor, and CD71, the transferrin receptor, as well as by the loss of the proliferative capacity. The functional effects have been monitored by assaying several cell functions, including generation of reactive oxygen metabolites and lysosomal-enzyme secretion, which appear during the cell-differentiation process, as well as by examining the underlying phospholipase-mediated signalling events.

MATERIALS AND METHODS

Materials

RA (all-*trans*), dexamethasone, *N*-formylmethionyl-leucyl-phenylalanine (FMLP), cytochalasin B, PMA, ATP and *p*-nitrophenyl *N*-acetyl- β -glucosaminide were purchased from

Abbreviations used: AA, arachidonic acid; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DMSO, dimethyl sulphoxide; FMLP, *N*-formylmethionyl-leucyl-phenylalanine; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; PMA, 4 β -phorbol 12-myristate 13-acetate; RA, retinoic acid.

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Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ionomycin was from Calbiochem (San Diego, CA, U.S.A.). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR, U.S.A.). RPMI 1640, C199, fetal-calf serum and glutamine were from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Gentamicin and penicillin were from Laboratorios Llorente (Madrid, Spain). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was from Dako-patts (Glostrup, Denmark). Phosphatidylethanol was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Silica-gel plates were from Merck (Darmstadt, Germany). [^3H]Thymidine, [^3H]arachidonic acid ([^3H]AA), [^3H]palmitic acid and *myo*-[^3H]inositol were from Amersham Iberica (Madrid, Spain). All other chemicals were of the best quality available from Merck and Sigma.

The anti-CD11b Bear-1 mAb [23] was generously given by Dr. J. E. De Vries (Unicet, Lyon, France). The anti-CD71 mAb [24] and the P3X63 myeloma culture supernatant, used as a negative control, were kindly provided by Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain).

Cells

The human promyelocytic HL-60 cell line was cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal-calf serum, 2 mM glutamine, 100 units/ml penicillin and 24 $\mu\text{g}/\text{ml}$ gentamicin at 37 °C in a humidified atmosphere of air/ CO_2 (19:1). HL-60 cells were induced to differentiate towards the granulocytic lineage by incubation with 1.3% (v/v) DMSO or 1 μM RA, in the absence or presence of 0.1 μM dexamethasone, freshly prepared before use.

Immunofluorescence flow cytometry

The cell surface expression of CD11b and CD71 antigens was analysed by immunofluorescence flow cytometry as previously described [25]. Fluorescence data were collected in log scale. Results were shown as cell number versus fluorescence intensity, cells incubated with P3X63 myeloma culture supernatant being used as a negative control.

Proliferation assay

Cells ($10^5/\text{ml}$) were incubated in 96-well plates with 250 μl of culture medium containing the differentiating agents in the absence or presence of dexamethasone. Cells were pulsed with 0.5 μCi of [^3H]thymidine/ml for 12 h, before harvesting in glass-fibre filters, and [^3H]thymidine incorporation was measured in a liquid-scintillation counter. Results were expressed as percentage of proliferation with respect to control values for untreated cells (100%).

Production of oxygen metabolites

The generation of superoxide anion was measured at 550 nm by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* in a discontinuous assay as previously described [26]. Results were expressed as nmol of reduced cytochrome *c*/10 min per 10^6 cells.

Production of H_2O_2 was monitored with the fluorescent indicator DCFH-DA by a modification of a previously described cytofluorimetric assay [27]. Cells ($5 \times 10^6/\text{ml}$) were loaded with 5 μM of DCFH-DA in Hepes/glucose buffer (10 mM Hepes, 150 mM NaCl, 5 mM KOH, 1.2 mM MgCl_2 , 1.3 mM CaCl_2 and 5.5 mM glucose, pH 7.4) for 15 min at 37 °C and resuspended in

the same buffer at 10^6 cells/ml. After addition of the stimuli, samples were incubated for a further 30 min at 37 °C. Reactions were stopped by placing the tubes on ice and fluorescence was measured in an EPICS flow cytometer (Coulter Cientifica, Móstoles, Spain). Fluorescence data were represented as cell number versus fluorescence intensity in a linear scale.

Lysosomal-enzyme release

This was assessed by measuring *N*-acetyl- β -glucosaminidase secretion. Cells ($10^6/\text{ml}$) were preincubated with 5 μg of cytochalasin B/ml in Hepes/glucose buffer for 5 min at 37 °C, transferred to tubes containing the stimuli and incubated for a further 10 min at 37 °C. Then the tubes were placed on ice and centrifuged at 300 *g* for 5 min. Supernatants were kept and assayed for *N*-acetyl- β -glucosaminidase activity, as previously described [28]. Results were expressed as percentage of enzyme release with respect to total enzyme content, and corrected for enzyme release from unstimulated cells.

Measurement of [^3H]inositol phosphates

Cells were cultured in RPMI medium containing 1.3% DMSO in the absence or presence of 0.1 μM dexamethasone. At day 2 of treatment, cells ($10^6/\text{ml}$) were transferred to C199 medium supplemented with antibiotics, 2 mM glutamine, 5 $\mu\text{g}/\text{ml}$ transferrin, 5 units/ml insulin and 1.3% DMSO, in the absence or presence of 0.1 μM dexamethasone, and incubated overnight with 1 $\mu\text{Ci}/\text{ml}$ *myo*-[^3H]inositol, as previously reported [29]. Then, cells ($5 \times 10^6/\text{ml}$) were preincubated at 37 °C for 10 min in Hepes/glucose buffer containing 10 mM LiCl and incubated for a further 15 min at 37 °C with the stimuli. Total inositol phosphates and total phospholipids were recovered as previously described [16]. Results were shown as percentage of total inositol phosphates accumulation with respect to total lipids, and corrected for values from unstimulated control cells.

Measurement of [^3H]AA release

Cells ($10^6/\text{ml}$) were labelled overnight with 0.25 μCi of [^3H]AA/ml in culture medium. As previously reported [30], cells ($10^7/\text{ml}$) were preincubated in Hepes/glucose buffer for 5 min at 37 °C with 5 $\mu\text{g}/\text{ml}$ cytochalasin B, and then with the stimuli for additional 15 min at 37 °C. Reaction was stopped by addition of 500 μl of ice-cold PBS and centrifugation at 300 *g* for 5 min. Supernatants were transferred to scintillation vials and counted for radioactivity. Results were shown as percentage of AA released with respect to total AA content of cells and corrected for values from unstimulated control cells.

Measurement of phospholipase D (PLD) activity

PLD activity was measured by the formation of phosphatidylethanol [31]. Cells ($10^6/\text{ml}$) were labelled overnight in culture medium with 5 $\mu\text{Ci}/\text{ml}$ of [^3H]palmitic acid. Subsequently, cells were preincubated in Hepes/glucose buffer in the presence of 5 μl of ethanol/ml and 5 μg of cytochalasin B/ml for 5 min at 37 °C, and then incubated for a further 30 min at 37 °C with the stimuli. Reactions were stopped by adding 750 μl of chloroform/methanol/4 M HCl (50:100:1, by vol.). Then 250 μl of chloroform and 250 μl of 0.1 M HCl were added to form two phases. After centrifugation at 500 *g* for 5 min, the organic phases were recovered, dried and spotted on silica-gel plates. Plates were developed with ethyl acetate/iso-octane/acetic acid (11:5:2, by vol.), dried and rechromatographed once in the same system. Lipids were localized by iodine staining and identified by comparison with authentic standards. The spots corresponding

Figure 1 Effect of dexamethasone on CD11b and CD71 cell-surface expression in HL-60 cells treated with DMSO or RA

Undifferentiated HL-60 cells or cells treated with 1.3% DMSO or 1 μ M RA were cultured in the absence (----) or presence (—) of 0.1 μ M dexamethasone. (a) Effect of dexamethasone on day 4 of the different treatments. Profiles represent relative cell number versus relative fluorescence intensity (on a log scale) of cells labelled with anti-CD11b mAb or anti-CD71 mAb. (b) Effect of dexamethasone on the percentage of HL-60 cells expressing CD11b and CD71 at the cell surface. HL-60 cells were treated with DMSO or RA in the absence ($\square$) or in the presence ($\blacksquare$) of 0.1 μ M dexamethasone. Undifferentiated HL-60 cells were also cultured in the absence ($\triangle$) or presence ($\blacktriangle$) of 0.1 μ M dexamethasone. Flow-cytometric analysis for CD11b and CD71 cell-surface expression was carried out during each day of treatment. Results are expressed as percentage of positive cells expressing these antigens versus incubation time.

to phosphatidylethanol, phosphatidic acid and phospholipids were scraped off and counted for radioactivity. Results were shown as percentage of phosphatidylethanol formation with respect to that of unstimulated control cells (100%), after correction of recovery from total lipids.

RESULTS

Alteration of CD11b and CD71 expression during DMSO- and RA-induced granulocytic HL-60 cell differentiation by dexamethasone

DMSO- or RA-induced granulocytic cell differentiation is characterized by an increase in CD11b and a decrease in CD71 cell-surface expression, as previously described [2,4]. Dexamethasone

treatment enhanced the RA-induced increase in CD11b expression, but diminished the DMSO-induced increase in CD11b expression. However, dexamethasone enhanced both the DMSO- and RA-induced decreases in CD71 cell-surface expression, as shown in Figure 1(a). Figure 1(b) shows the expression of cell-surface CD11b and CD71 throughout the whole DMSO- and RA-induced cell-differentiation period in the absence or presence of dexamethasone. Dexamethasone alone did not have any effect on CD11b and CD71 cell-surface expression in undifferentiated HL-60 cells, even after prolonged incubation times (Figure 1b).

Disappearance of CD71 has been associated with loss of cell proliferative capacity [2]. As dexamethasone modified CD71 cell-surface antigen expression, the action of dexamethasone on HL-60 proliferation was also studied. Inhibition of HL-60 cell proliferation was observed after DMSO and RA treatments, the inhibitory effect being more rapid in DMSO-treated cells (70 and 84% inhibition at days 2 and 4 of treatment respectively) than in RA-treated cells (9 and 74% inhibition at days 2 and 4 of treatment respectively) (Table 1). Dexamethasone potentiated slightly the inhibitory response induced by RA (increased inhibition of 24 and 8% at days 2 and 4 respectively, as compared with RA-treated cells). Nevertheless, dexamethasone hardly affected the DMSO-induced inhibition of cell proliferation (Table 1). Dexamethasone alone did not have any effect on the proliferative capacity of HL-60 cells when added in the dose range 0.1 nM–10 μ M after 4 days of treatment (results not shown).

Table 1 Effect of dexamethasone on [^{3} H]thymidine incorporation in DMSO- and RA-treated HL-60 cells

Cells (2.5×10^4) were incubated without or with 1.3% (v/v) DMSO or 1 μ M RA, in the absence or presence of 0.1 μ M dexamethasone (Dex), for 2 or 4 days, and 0.125 μ Ci of [^{3} H]thymidine was added 12 h before cell harvesting. Results are represented as percentage of proliferation with respect to control undifferentiated HL-60 cells. Data are means $\pm$ S.D. of triplicates of a representative experiment.

Treatment	[^{3} H]Thymidine incorporation (% of control)	
	2 days	4 days
Undifferentiated	100.0	100.0
Undifferentiated + Dex	109.2 $\pm$ 1.8	123.0 $\pm$ 3.7
1 μ M RA	91.2 $\pm$ 1.8	26.4 $\pm$ 1.9
1 μ M RA + Dex	67.7 $\pm$ 1.9	18.9 $\pm$ 1.9
1.3% DMSO	29.8 $\pm$ 0.9	15.8 $\pm$ 0.9
1.3% DMSO + Dex	35.1 $\pm$ 0.9	11.3 $\pm$ 0.9

Dexamethasone affects lysosomal-enzyme secretion in undifferentiated HL-60 cells and in DMSO- or RA-induced granulocytic differentiating HL-60 cells

Differentiated HL-60 cells become responsive to FMLP due to the induction of chemotactic-peptide receptors [32]. Dexamethasone enhanced the secretory responses to 1 μ M FMLP and

Table 2 Effect of dexamethasone on receptor- and non-receptor-mediated *N*-acetyl- β -glucosaminidase secretion in undifferentiated and DMSO- or RA-treated HL-60 cells

Undifferentiated HL-60 cells and RA- and DMSO-treated HL-60 cells for 2 or 4 days, in the absence or presence of 0.1 μ M dexamethasone (Dex), were stimulated with 100 μ M ATP, 1 μ M FMLP, 20 ng/ml PMA, 1 μ M ionomycin (ION) or with a combination of PMA and ionomycin as described in the Materials and methods section. Results are expressed as percentage of released enzyme from total cell enzyme content and corrected for enzyme release from unstimulated cells. Enzyme release from unstimulated cells was in the range of 5–15% of total cell enzyme content, irrespective of cell treatment. Data are means $\pm$ S.D. of triplicates of a representative experiment. N.D., not determined.

Time	Treatment	Percentage of stimulated <i>N</i> -acetyl- β -glucosaminidase release				
		ATP	FMLP	PMA	ION	ION + PMA
2 days	Undifferentiated	5.8 $\pm$ 0.2	0.6 $\pm$ 0.1	N.D.	N.D.	N.D.
	Undifferentiated + Dex	18.2 $\pm$ 0.3	2.5 $\pm$ 0.3	N.D.	N.D.	N.D.
	1 μ M RA	4.2 $\pm$ 0.7	0.8 $\pm$ 0.1	N.D.	N.D.	N.D.
	1 μ M RA + Dex	12.3 $\pm$ 3.1	3.9 $\pm$ 0.2	N.D.	N.D.	N.D.
	1.3% DMSO	12.3 $\pm$ 0.1	8.9 $\pm$ 0.5	N.D.	N.D.	N.D.
	1.3% DMSO + Dex	22.7 $\pm$ 3.0	35.4 $\pm$ 0.4	N.D.	N.D.	N.D.
4 days	Undifferentiated	N.D.	N.D.	0.6 $\pm$ 0.1	1.3 $\pm$ 0.2	12.4 $\pm$ 0.1
	Undifferentiated + Dex	N.D.	N.D.	13.4 $\pm$ 0.2	5.3 $\pm$ 0.5	27.9 $\pm$ 0.1
	1 μ M RA	7.8 $\pm$ 0.5	8.5 $\pm$ 0.1	6.0 $\pm$ 0.5	8.0 $\pm$ 0.3	18.0 $\pm$ 0.1
	1 μ M RA + Dex	10.1 $\pm$ 0.8	21.4 $\pm$ 0.2	14.4 $\pm$ 0.6	15.3 $\pm$ 0.4	37.3 $\pm$ 1.0
	1.3% DMSO	31.8 $\pm$ 5.6	18.2 $\pm$ 0.1	25.2 $\pm$ 2.0	44.4 $\pm$ 0.5	44.8 $\pm$ 1.5
	1.3% DMSO + Dex	39.8 $\pm$ 0.1	44.0 $\pm$ 4.0	26.8 $\pm$ 2.0	42.9 $\pm$ 1.0	52.6 $\pm$ 0.5

Table 3 Effect of dexamethasone on superoxide anion production in RA- or DMSO-induced granulocytic HL-60 differentiating cells

Cells treated for 2 or 4 days with 1 μ M RA or 1.3% DMSO, in the absence or presence of 0.1 μ M dexamethasone (Dex), were stimulated with 1 μ M FMLP or 50 ng/ml PMA, and the generation of superoxide anion was assayed as described in the Materials and methods section. Superoxide anion production is expressed in nmol of cytochrome *c* reduced/10 min per 10⁶ cells and the results are shown as means $\pm$ S.D. of triplicates of a representative experiment.

Treatment	Superoxide anion production (nmol of cytochrome <i>c</i> reduced/10 min per 10 ⁶ cells)			
	FMLP		PMA	
	2 days	4 days	2 days	4 days
1 μ M RA	0.6 $\pm$ 0.5	2.2 $\pm$ 0.3	9.4 $\pm$ 0.6	11.2 $\pm$ 0.4
1 μ M RA + Dex	6.2 $\pm$ 0.4	6.9 $\pm$ 0.7	16.6 $\pm$ 0.5	16.2 $\pm$ 0.3
1.3% DMSO	1.6 $\pm$ 0.6	4.3 $\pm$ 0.5	12.1 $\pm$ 0.4	20.6 $\pm$ 1.2
1.3% DMSO + Dex	6.3 $\pm$ 0.5	13.1 $\pm$ 1.9	8.6 $\pm$ 1.2	13.0 $\pm$ 0.6

100 μ M ATP in both DMSO- and RA-treated cells (Table 2). Undifferentiated HL-60 cells, treated for 2 days with dexamethasone alone, also showed an increased secretory response to ATP and a slight response to FMLP (Table 2). We further studied the effect of dexamethasone treatment on the secretory response induced by non-receptor-mediated stimuli. Thus we examined the effect of dexamethasone on the secretory response to 20 ng/ml PMA, 1 μ M ionomycin or the combination of both agents in undifferentiated HL-60 cells as well as in the DMSO- and RA-induced granulocytic differentiating cells. Although PMA and ionomycin were weak secretagogues in undifferentiated HL-60 cells, the combination of both stimuli induced a synergistic effect. Dexamethasone largely enhanced the secretory response to these agents in undifferentiated HL-60 cells (Table 2). RA-treated cells showed an increased response to these stimuli, similar to that observed in undifferentiated cells, and dexamethasone enhanced these secretory responses (Table 2). DMSO-treated cells showed the highest secretory response to the same doses of PMA or

ionomycin, but in this case the combination of both stimuli did not induce a potentiation of the secretory response like that observed in undifferentiated cells (Table 2), probably because the response to ionophore alone was already maximum. In this case, dexamethasone did not modify the secretory response to PMA, ionomycin or the combination of both stimuli in DMSO-treated cells (Table 2).

Differential effect of dexamethasone on the respiratory burst activity in DMSO- and RA-treated HL-60 cells

HL-60 cells induced to differentiate towards granulocytes develop the capacity to generate reactive oxygen metabolites [6]. Although HL-60 cells have purinergic receptors, these are not coupled to the generation of oxidative response when HL-60 cells are differentiated with DMSO or RA [33]. We examined whether dexamethasone treatment affected the capacity of HL-60 cells treated with RA or DMSO for 2 and 4 days to generate superoxide anion (Table 3) and H₂O₂ (Figure 2), with 50 ng/ml PMA or 1 μ M FMLP as stimuli. Dexamethasone enhanced the oxidative responses triggered by FMLP or by PMA (Table 3 and Figure 2a) in RA-treated cells, and it also enhanced the capacity to generate oxygen metabolites in response to FMLP in DMSO-treated cells (Table 3 and Figure 2b). However, dexamethasone inhibited the oxidative response to PMA in DMSO-treated cells (Table 3 and Figure 2b). Undifferentiated cells were unable to generate an oxidative response to any stimulus, and dexamethasone treatment did not modify this pattern (results not shown).

Dexamethasone affects differentially phospholipase activities in undifferentiated and in DMSO- or RA-treated HL-60 cells

It has been postulated that dexamethasone produces its effects through synthesis of phospholipase inhibitors [12]. Nevertheless, we found that secretion of lysosomal enzymes and generation of oxygen metabolites, two cellular functions probably mediated by phospholipases [34], were enhanced in dexamethasone-treated cells. In this context, we analysed the effect of dexamethasone on the activation of phospholipase C (PLC), phospholipase A₂ (PLA₂) and PLD by distinct stimuli. Dexamethasone-treated cells showed an increased inositol phosphate accumulation in

Figure 2 Effect of dexamethasone on H_2O_2 production in RA- or DMSO-induced granulocytic differentiating cells

Cells were treated for 4 days with $1 \mu\text{M}$ RA (a) or 1.3% DMSO (b) in the absence or presence of $0.1 \mu\text{M}$ dexamethasone (DEX). H_2O_2 production was measured by using the fluorescent probe DCFH-DA in unstimulated cells (—) or in cells stimulated with $1 \mu\text{M}$ FMLP or 50 ng/ml PMA (---) for 30 min at 37°C . Data are shown as cell number versus fluorescence intensity on a linear scale. Numbers at the upper right part of each profile indicate net H_2O_2 production for each treatment, in arbitrary fluorescence units.

Figure 3 Dexamethasone effects on total inositol phosphates accumulation and *N*-acetyl- β -glucosaminidase release in DMSO- and RA-induced differentiating HL-60 cells

HL-60 cells were treated for 2 days with $1 \mu\text{M}$ RA or 1.3% DMSO, in the absence ($\circ$, $\square$) or presence ($\bullet$, $\blacklozenge$) of $0.1 \mu\text{M}$ dexamethasone, and then stimulated with the indicated concentrations of FMLP or ATP at 37°C for 15 min. Total inositol phosphates accumulation and *N*-acetyl- β -glucosaminidase release were measured as described in the Materials and methods section. The total inositol phosphate accumulation in unstimulated cells with or without dexamethasone was 228 ± 30 and 240 ± 54 c.p.m., which corresponded to $0.50 \pm 0.07\%$ and $0.53 \pm 0.18\%$ for RA-treated cells, and 1131 ± 16 and 841 ± 37 c.p.m., which corresponded to $1.97 \pm 0.03\%$ and $1.46 \pm 0.06\%$ for DMSO-treated cells. Results are expressed as means $\pm$ S.D. of triplicates of a representative experiment.

response to different concentrations of FMLP or ATP in DMSO- or RA-induced differentiating HL-60 cells, and this enhanced response was correlated with an increased lysosomal enzyme secretion (Figure 3). PLA_2 activity was measured by AA release from [^3H]AA-prelabelled cells. Unstimulated AA release was higher in undifferentiated HL-60 cells than in dexamethasone-treated cells ($3.38 \pm 0.01\%$ versus $1.36 \pm 0.09\%$). AA release was not detected when undifferentiated cells were stimulated with $100 \mu\text{M}$ ATP, $1 \mu\text{M}$ ionomycin or 20 ng/ml PMA. However, dexamethasone-treated cells responded to these stimuli, releasing

AA (Table 4). RA-treated cells showed similar values of unstimulated AA release regardless of dexamethasone treatment ($2.42 \pm 0.2\%$ versus $2.26 \pm 0.43\%$). However, although AA release was hardly detected in stimulated RA-treated cells, this response became apparent in dexamethasone-treated cells (Table 4). DMSO-treated cells showed similar unstimulated release in the presence or absence of dexamethasone ($1.97 \pm 0.14\%$ versus $2.19 \pm 0.08\%$), but a remarkable increase in stimulated AA release was observed when compared with undifferentiated or RA-treated cells. Nevertheless, dexamethasone treatment did not

Table 4 Effect of dexamethasone on PLA₂ and PLD activity in undifferentiated, RA- and DMSO-treated HL-60 cells

Undifferentiated HL-60 cells or cells treated with RA or DMSO for 4 days in the absence or presence of 0.1 μ M dexamethasone (Dex) were stimulated with 100 μ M ATP, 1 μ M FMLP, 20 ng/ml PMA, 1 μ M ionomycin (ION) or with the combination of both PMA and ionomycin (ION + PMA) at 37 °C. PLA₂ was measured as AA release after 15 min incubation at 37 °C with the distinct stimuli as described in the Materials and methods section. Unstimulated AA release in the absence or presence of dexamethasone was respectively 4562 $\pm$ 10 and 1832 $\pm$ 310 c.p.m. for control untreated cells, 1539 $\pm$ 317 and 1431 $\pm$ 125 c.p.m. for RA-treated cells, and 1937 $\pm$ 139 and 2151 $\pm$ 182 c.p.m. for DMSO-treated cells. PLD was measured as phosphatidylethanol formation after 30 min incubation at 37 °C with the distinct stimuli as described in the Materials and methods section. The 100% value of phosphatidylethanol formation was 1221 $\pm$ 271 and 3825 $\pm$ 394 c.p.m. for control untreated cells, 1773 $\pm$ 128 and 2165 $\pm$ 35 c.p.m. for DMSO-treated cells and 1272 $\pm$ 20 and 1258 $\pm$ 22 c.p.m. for RA-treated cells in the absence or presence of dexamethasone respectively. Results are expressed as the mean $\pm$ S.D. of triplicates of a representative experiment.

Treatment	PLD activity (phosphatidylethanol accumulation; % of control)				PLA ₂ activity (AA release; % of total)				
	ATP	FMLP	PMA	ION	ATP	FMLP	PMA	ION	ION + PMA
Undifferentiated	114 $\pm$ 11	112 $\pm$ 19	588 $\pm$ 15	189 $\pm$ 13	—	—	—	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01
Undifferentiated + Dex	108 $\pm$ 1	100 $\pm$ 16	292 $\pm$ 9	204 $\pm$ 48	0.10 $\pm$ 0.01	—	0.03 $\pm$ 0.05	0.29 $\pm$ 0.05	0.35 $\pm$ 0.01
1 μ M RA	100 $\pm$ 10	100 $\pm$ 8	169 $\pm$ 1	100 $\pm$ 8	—	—	—	0.06 $\pm$ 0.02	0.09 $\pm$ 0.03
1 μ M RA + Dex	168 $\pm$ 7	162 $\pm$ 2	296 $\pm$ 9	178 $\pm$ 3	1.35 $\pm$ 0.02	0.35 $\pm$ 0.08	0.12 $\pm$ 0.34	0.63 $\pm$ 0.41	0.42 $\pm$ 0.51
1.3% DMSO	147 $\pm$ 9	221 $\pm$ 10	1467 $\pm$ 79	641 $\pm$ 27	0.46 $\pm$ 0.01	2.02 $\pm$ 0.03	0.53 $\pm$ 0.11	1.80 $\pm$ 0.02	2.73 $\pm$ 0.03
1.3% DMSO + Dex	126 $\pm$ 17	200 $\pm$ 1	470 $\pm$ 14	446 $\pm$ 4	0.77 $\pm$ 0.15	1.93 $\pm$ 0.06	0.41 $\pm$ 0.02	1.69 $\pm$ 0.04	3.22 $\pm$ 0.08

affect significantly the PLA₂ activation induced by distinct stimuli in DMSO-treated HL-60 cells (Table 4).

We measured phosphatidylethanol formation in response to 100 μ M ATP, 1 μ M FMLP, 20 ng/ml PMA or 1 μ M ionomycin, in the presence or absence of dexamethasone. In undifferentiated HL-60 cells, PLD was activated by PMA and ionomycin (5.9- and 1.9-fold increase with respect to unstimulated control cells, respectively), but not by ATP or FMLP. Dexamethasone treatment decreased the response to PMA (50% inhibition), whereas the response to ionomycin was not affected (Table 4). In DMSO-treated cells, PLD activity was markedly higher than in undifferentiated cells. In these DMSO-treated cells, dexamethasone strongly inhibited the PLD activity upon PMA stimulation (68% inhibition), slightly inhibited the PLD response to ionomycin (30% inhibition), and showed no significant effect on the weak receptor-mediated responses to ATP or to FMLP (Table 4). In contrast, dexamethasone significantly enhanced PLD activation by all stimuli used in RA-treated cells (Table 4).

DISCUSSION

Glucocorticoids have been reported to affect granulopoiesis [14,15] as well as differentiation and functional parameters of neutrophils [8–10,35,36] and of some myeloid cell lines [19–22]. Here we report that dexamethasone affects differentially several immunophenotypic and functional parameters as well as effector systems in HL-60 cells, depending on the maturation stage of the cells and on the agent used to induce granulocytic differentiation.

Dexamethasone enhances the secretory response and PLA₂ activity in undifferentiated HL-60 cells

We have found that dexamethasone by itself did not modify the cell-surface expression of the CD11b and CD71 antigens and failed to affect cell proliferation or to induce the appearance of phagocytic functions, such as the capacity to generate an oxidative response, in undifferentiated HL-60 cells. Thus, dexamethasone by itself does not induce HL-60 cell differentiation. Nevertheless, dexamethasone enhanced the secretory response induced by ATP and by non-receptor-mediated stimuli, such as PMA, ionomycin or the combination of both stimuli. It has been postulated that the secretory response seems to involve activation

of PLC, PLA₂ or PLD [34]. Undifferentiated cells did not show AA release upon cell stimulation. However, it is noteworthy that dexamethasone treatment induced an AA-release response to several receptor- and non-receptor-mediated stimuli, suggesting that the induction of PLA₂ activity could be responsible for the enhancement of the secretory response by dexamethasone. This view is further supported by the fact that PMA-stimulated PLD activity was inhibited by dexamethasone.

Differential effects of dexamethasone on RA- and DMSO-induced differentiating HL-60 cells

We studied the effect of dexamethasone in the granulocytic differentiation of HL-60 cells induced by RA or DMSO. In RA-induced differentiating cells, dexamethasone enhanced the appearance of CD11b and the disappearance of CD71 antigens as well as the inhibition of proliferative capacity. Furthermore, we found that the functional maturation of RA-induced granulocytic HL-60 cells was greatly accelerated by dexamethasone treatment. Contrarily to the immunosuppressive effects of glucocorticoids reported in other systems [10,20–22], we found that PLC, PLA₂ and PLD activities were enhanced by dexamethasone in RA-treated cells, suggesting that the increase in secretory and oxidative activities could be mediated by the increase in these phospholipase activities. These data indicate that dexamethasone potentiates the granulocytic differentiating effect of RA, in part by enhancing the activities of the underlying signal-transducing mechanisms.

In DMSO-induced differentiating HL-60 cells, dexamethasone showed differential effects. Regarding antigen expression, dexamethasone inhibited CD11b appearance, but it enhanced CD71 disappearance. As for functional responses, dexamethasone produced an increase in the oxidative and secretory responses mediated through stimulation of FMLP or ATP receptors as well as in the FMLP- or ATP-induced activation of PLC activity, whereas ATP-induced PLA₂ activation was slightly increased and PLD activation was unchanged. These data indicate that the increase in the receptor-mediated secretory and oxidative responses might involve PLC/PLA₂ activities rather than PLD. Non-receptor-mediated secretory responses to PMA or ionomycin were not modified by dexamethasone treatment. However, dexamethasone inhibited the oxidative response to PMA. There-

fore, we studied the action of dexamethasone on phospholipase activities stimulated by PMA to elucidate the underlying differences in the effects of dexamethasone on the oxidative response triggered by PMA or FMLP. PMA-stimulated PLA₂ activity was unchanged, whereas PMA-stimulated PLD activity was inhibited by dexamethasone treatment. These results suggest that PLD activation is coupled to superoxide generation in cells stimulated with PMA [37–39]. The opposite effects induced by dexamethasone on the oxidative responses triggered by FMLP or PMA indicate that different signalling pathways might be involved in oxygen-metabolite generation induced by FMLP or PMA, as previously suggested [40], being affected in different ways by dexamethasone. Thus the inhibitory effect of dexamethasone on both PMA-induced oxidative response and PLD activity suggests that PLD activation would be more relevant than PLA₂ or PLC in eliciting the oxidative response in PMA-stimulated cells.

The data herein reported indicate that, although phospholipase activation mediates the signalling of secretory and oxidative responses, as previously suggested [29,34,39], the contribution of the different signalling pathways to cellular responses might be different, depending on the differentiation stage of cells. Both RA and DMSO increase to a different extent the secretory and oxidative responses with respect to undifferentiated cells, but the enhancement of PLD and PLA₂ activities during DMSO-induced differentiation is not observed in RA-treated cells. Furthermore, the fact that dexamethasone affects differently the oxidative and secretory responses to PMA in RA- or DMSO-treated cells supports this concept. In this context, it has been reported that the levels of PKC isoenzymes [41] increase during HL-60 differentiation induced by RA or DMSO, with a higher increase in α -isoform in DMSO-treated cells with respect to RA-treated cells. The differential responsiveness of PKC to distinct diacylglycerol molecular species or AA underscores the significance of the existence of multiple PKC isoforms that may vary considerably with their sensitivity to various activating agents, including PMA or diacylglycerol [42]. These processes might also contribute to the observed differential effect of dexamethasone on several responses. In agreement with other studies in U937 cells [20–22], we have found that dexamethasone affects mainly the functional responses of HL-60 cells, but, contrarily to what might be expected, dexamethasone induces a selective enhancement of functional responses and signalling mechanisms, depending on the agent used to induce differentiation [33], indicating the involvement of distinct signal-transduction pathways in the development of mature granulocytes. The enhanced effect of dexamethasone on receptor-mediated responses could be due, in part, to an increase in the number of receptors as previously reported for FMLP receptors in cells differentiated with dimethyl formamide or DMSO [32]. However, it is worthwhile to note that dexamethasone does not induce an overall up-regulation of receptors. In this context, inhibition of Fc receptor number upon dexamethasone treatment has been reported in HL-60 cells [43]. Moreover, the increase in the receptor number does not explain the changes in non-receptor-mediated responses and the selective responses to different stimuli, suggesting that the dexamethasone effect is not due to a direct action on the responsible enzymes for a particular function, but to alteration in the coupling between receptor and effector systems.

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