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EXPERIMENTAL STUDY

Opposing effects of dehydroepiandrosterone and dexamethasone on the generation of monocyte-derived dendritic cells

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Abstract

Background: Dehydroepiandrosterone (DHEA) has been suggested as an immunostimulating steroid hormone, of which the effects on the development of dendritic cells (DC) are unknown. The effects of DHEA often oppose those of the other adrenal glucocorticoid, cortisol. Glucocorticoids (GC) are known to suppress the immune response at different levels and have recently been shown to modulate the development of DC, thereby influencing the initiation of the immune response. Variations in the duration of exposure to, and doses of, GC (particularly dexamethasone (DEX)) however, have resulted in conflicting effects on DC development.

Aim: In this study, we describe the effects of a continuous high level of exposure to the adrenal steroid DHEA (10^{-6} M) on the generation of immature DC from monocytes, as well as the effects of the opposing steroid DEX on this development.

Results: The continuous presence of DHEA (10^{-6} M) in GM-CSF/IL-4-induced monocyte-derived DC cultures resulted in immature DC with a morphology and functional capabilities similar to those of typical immature DC (T cell stimulation, IL-12/IL-10 production), but with a slightly altered phenotype of increased CD80 and decreased CD43 expression (markers of maturity).

The continuous presence of DEX at a concentration of 10^{-6} M in the monocyte/DC cultures resulted in the generation of plastic-adherent macrophage-like cells in place of typical immature DC, with increased CD14 expression, but decreased expression of the typical DC markers CD1a, CD40 and CD80. These cells were strongly reactive to acid phosphatase, but equally capable of stimulating T cell proliferation as immature DC. The production of IL-12 by these macrophage-like cells was virtually shut down, whereas the production of IL-10 was significantly higher than that of control immature DC.

Conclusion: The continuous presence of a high level of GC during the generation of immature DC from monocytes can modulate this development away from DC towards a macrophage-like cell. The combination of a low CD80 expression and a shutdown of IL-12 production suggests the possibility of DEX-generated cells initiating a Th2-biased response. These effects by DEX on DC development contrast with those by DHEA, which resulted in a more typical DC although possessing a phenotype possibly indicating a more mature state of the cell.

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Introduction

Dehydroepiandrosterone (DHEA) is quantitatively the most abundant adrenal steroid hormone in humans and other mammals (1, 2). The hormone is uniquely sulfated (DHEA-S) before entering the plasma, and the sulfated prohormone is converted to DHEA and its metabolites (3) in various peripheral tissues. No major endocrine functions have been ascribed to a direct action of DHEA-S and DHEA, although the hormones act as intermediaries in sex steroid synthesis (3). Both hormones, however, have been proposed as exerting important restoring effects on age-related processes, such as fat depot distrubution and neurodegeneration. These effects also include major stimulation of cells of the (aging) immune system (4–6). However, these effects of DHEA have also been disputed (7, 8).

Dendritic cells (DC) are antigen-presenting cells *par excellence*, and the only cells capable of stimulating naïve T cells and thus capable of initiating primary

immune responses (9). A well-accepted method of generating DC from monocytes is the culture of monocytes in the presence of the cytokines GM-CSF and IL-4 for 7 days (10). This procedure yields the so-called 'immature' DC, with a retained capability for uptake and processing of antigens but with a relatively low capability to stimulate T cells. A further exposure of 'immature' DC to pro-inflammatory stimuli (e.g. IL-1, lipopolysaccharide (LPS)) generates mature forms of the cell with an enhanced capability to stimulate T cells, but an almost lost capability for antigen uptake and processing (11).

There are no reports on the effects of DHEA on dendritic cell development. We previously reported that the exposure of monocytes to hormones (in particular to triiodothyronine) stimulated DC development from monocytes (12, 13). We now have tested and report here the effects of exposure of monocytes to DHEA, prior to, and during, their differentiation into immature DC under the influence of GM-CSF and IL-4.

In biological systems, the effects of DHEA-S and DHEA are often opposed by the other important adrenal steroid cortisol (14). The ratio DHEA/cortisol is abnormal in various pathological conditions characterized by immune dysfunction, such as after thermal injury, in AIDS, in rheumatoid arthritis and in tuberculosis (15–19). Although the suppressive effects of glucocorticoids on T cells, B cells, monocytes and macrophages have extensively been studied (reviewed in 20, 21), there is a growing, but still limited number of reports on the effects of glucocorticoids on the function and differentiation of DC (22–26). Data in these reports are inconsistent regarding effects on marker expression, T cell-stimulatory capacity and cytokine production. We therefore contrasted our DHEA experiments with dexamethasone (DEX) and tested the effect of this hormone on the process of the transition from monocyte to immature DC also.

Materials and methods

Isolation of monocytes from peripheral blood

Monocytes were isolated from the peripheral blood of healthy blood donors via well-accepted methods. Heparinized blood diluted with an equal volume of phosphate buffered saline (PBS) was distributed over Ficoll Isopaque (density 1.077 g/ml, Pharmacia, Uppsala, Sweden) and centrifuged for 15 min at 1000 g. Cells were collected from the interface and washed and then suspended in RPMI 1640 with 25 mM HEPES and L-glutamine (GIBCO, Life Technologies, Breda, The Netherlands), supplemented with 100U/ml penicillin G, 0.1 mg/ml streptomycin (both Seromed, Biochrom, Berlin, Germany) and 10% FCS (Bio Whitaker, USA) (hereafter known as RPMI 1640^+) added. This cell suspension was distributed over Percoll (density 1.063 g/ml, Pharmacia, Uppsala, Sweden), then centrifuged for 40 min at 400 g. Cells collected from the interface were washed and suspended in RPMI 1640^+. Monocyte purity was determined by non-specific esterase staining (NSE) (27). Cell suspensions containing 80% or more monocytes were frozen following standard procedures and stored in liquid nitrogen, providing a bank for experiments. Monocytes purified by elutriator centrifugation were also used (courtesy of CLB, Amsterdam), in order to confirm results obtained via Ficoll/Percoll gradient separation.

Culture of DC from peripheral blood monocytes

DC were obtained via the well-established method first described by Sallusto and Lanzavecchia (10). Briefly, monocytes were cultured for 1 week at 37 °C, in 5% CO_2 and 100% humidity at a concentration of 3×10^5 cells/ml in RPMI 1640^+ with 800 U/ml GM-CSF and 1000 U/ml IL-4. The cultures were fed every 2 days, by removing 500 μl culture fluid and replacing this with 1 ml of fresh medium containing cytokines. In order to test the effects of exposure to DHEA and DEX on the monocyte-to-DC transition, these hormones were added at an optimal concentration of 10^{-6} M to monocytes in RPMI 1640 culture medium (without FCS) and incubated for 30 min at 37 °C, 5% CO_2, 100% humidity after which FCS (10%), GM-CSF (800 U/ml) and IL-4 (1000 U/ml) were added to the culture. DHEA and DEX were also fed to the cultures every 2 days along with fresh medium and cytokines. All culture medium used was tested and found to be free of endotoxin. Both hormones were purchased free from endotoxin contamination.

Flow cytometry and immunocytochemistry

For analysis of marker expression by flow cytometry, all cell populations were stained by incubating for 10 min with mouse anti-human FITC- or PE-conjugated monoclonal antibodies, followed by three washes. The monoclonal antibodies used were My4 (CD14, Beckman Coulter, Hialeah, FL, USA), CD1a (Beckman Coulter, Hialeah, FL, USA), B7.1 (CD80, Becton Dickinson, San Jose, CA, USA), B7.2 (CD86, PharMingen, Los Angeles, CA, USA), CD 40 (Serotec, Oxford, UK), CD43 (Biosource, Camarillo, CA, USA) and CD83 (Immunotech, Marseilles, France). Immediately following the staining, cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA).

The reactivity of the various monocyte/DC populations to acid phosphatase was determined using cytospins prepared on a Cytospin apparatus (Nordic Immunological Laboratories, The Netherlands) Cytospins were air-dried, then incubated for acid phosphatase staining according to Katayama *et al.* (28), using naphtol AS-BI phosphate as substrate and hexarotised pararosanilin as coupling agent (37 °C, 30 min). Slides

were counterstained with hematoxylin. Preparations were mounted in DePex mounting medium (Gurr, BDH Ltd, Poole, UK).

Mixed leukocyte reaction

Allogeneic mixed leukocyte reactions (MLRs) were performed in order to measure the accessory capability of the various DC populations generated. Responder T lymphocytes were obtained from a healthy donor and isolated following standard procedures with Ficoll-isopaque, Percoll density gradient centrifugation, and nylon wool adherence (Leuko-Pak, Fenwall Laboratories, IL, USA). Non-adhering cells recovered were greater than 90% CD3 positive. A total of 1.5×10^5 responder cells were cultured in 96-well, flat-bottom microtitre plates (NUNC A/S International, Denmark) with different numbers of irradiated (2000 rad) stimulator cells (monocytes or DC) to achieve stimulator-to-T cell ratios of 1:5, 1:10, 1:20 and 1:40. The culture medium used was RPMI 1640 with 25 mM HEPES and L-glutamine, supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 10% human A^+ serum, in a total volume of 200 μl per well. The controls used were monocytes or DC alone, and lymphocytes in the presence of 10–50 μg/ml phytohemagglutinin (PHA) (Wellcome Diagnostics, Zeist, The Netherlands). Cultures were performed in triplicate. On day 5, thymidine incorporation was assayed by adding 0.5 μCi [^{3}H]-thymidine to each well. Cells were harvested 16 h later and the radioactivity counted on an LKB 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland).

IL-12/IL-10 production

The DC were placed in 24-well plates (NUNC) at a concentration of 5×10^5 cells/ml and cultured for 24 h in RPMI$^+$ containing ultraglutamine (2 mM, BioWhitaker), penicillin/streptomycin (100 U/ml, 100 μg/ml, BioWhitaker) and serum free medium supplement (Pepro Tech, Rocky Hill, NJ, USA). To stimulate IL-10 production, the culture fluid contained *Staphylococcus aureus* cowan 1 strain (SAC) (1:5000, Calbiochem, La Jolla, CA, USA). The IL-12 production was stimulated by SAC (1:5000) and γ-IFN (1000 U/ml, Biomedical Primate Research Centre, Rijswijk, The Netherlands). The production was measured by ELISA as indicated by the manufacturer (for IL-10, ELISA Pelikine, CLB, Amsterdam, The Netherlands; IL-12, Eli-pair, Diaclone, Besançon, France).

Results

DHEA and DEX influence the phenotype of DC in culture

Dose–response curves were carried out to determine the optimal concentration of DHEA to add to the monocytes in culture. On the basis of our previous experience using various hormones to generate veiled cells (12, 13), the optimal concentration was determined to be that which resulted in the greatest number of cells displaying a veiled morphology. The optimal concentration for DHEA was found to be 10^{-6} M. In the case of DEX, we had previously carried out dose–response curves and have shown that although concentrations of 10^{-9} M were already effective, a concentration of 10^{-6} M added to DC obtained from bronchial alveolar lavage was optimal for obtaining a decreased function as well as a decrease in co-stimulatory molecule expression (29). We have therefore used 10^{-6} M DHEA and 10^{-6} M DEX in our subsequent experiments.

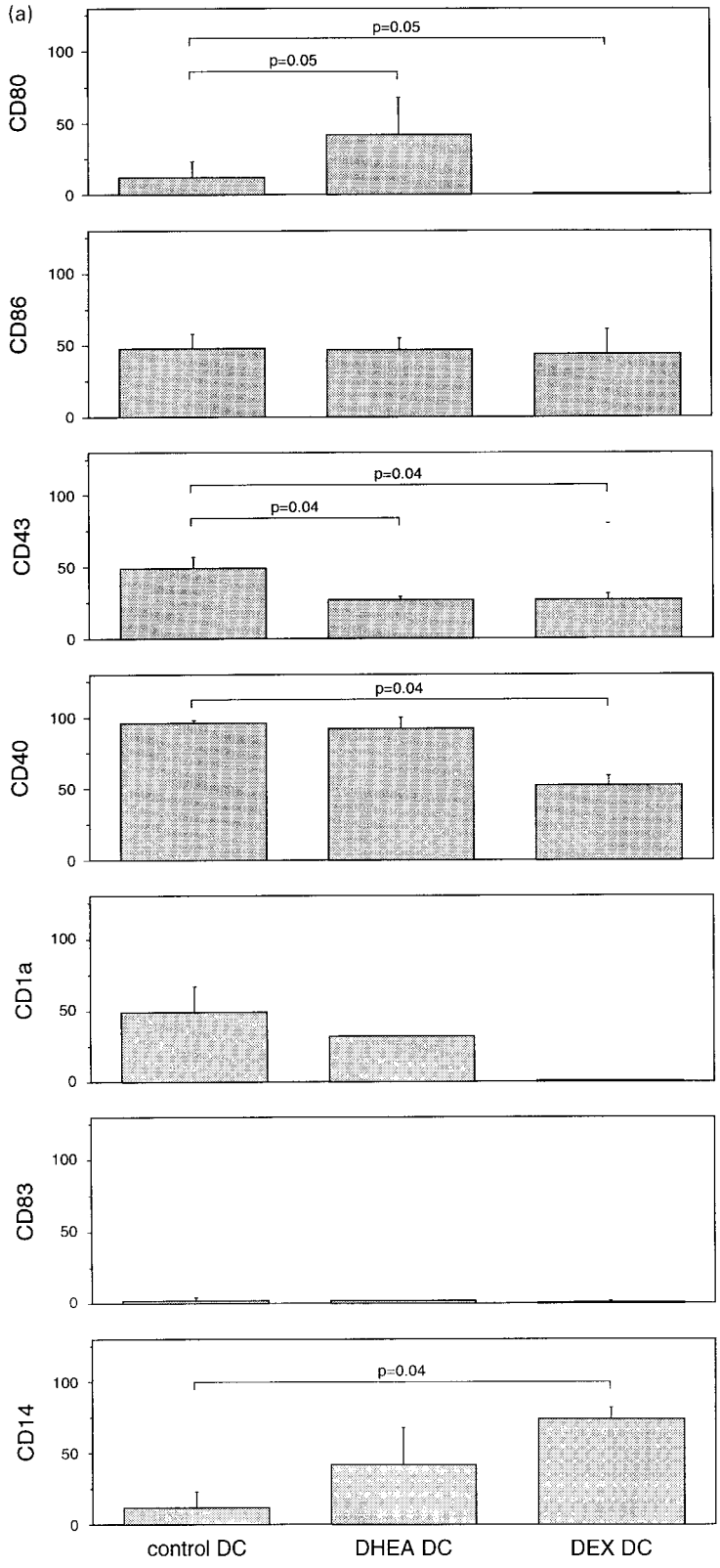
The addition of DHEA and DEX to the cultures resulted in the generation of DC with significantly different phenotypes than those generated with cytokines alone (Fig. 1), while not affecting cell viability or cell survival, as determined by cell recovery numbers and trypan blue exclusion.

Adding DHEA to the cultures resulted in a DC population with a marked upregulation of the co-stimulatory molecule CD 80 ($45 \pm 9\%$ vs $22 \pm 8\%$ positive cells in the control culture, $P = 0.05$). This was contrasted by a sharp downregulation of CD80 when DEX was added ($1 \pm 0\%$ vs. $22 \pm 8\%$ positive cells in the control culture, $P = 0.05$). Moreover, in the presence of DEX the morphology of the cells had changed, appearing as largely adherent macrophage-like cells with blunted cytoplasmic processes. The expression of the co-stimulatory molecule CD86 remained constant, regardless of exposure to either DHEA or DEX.

Both hormones decreased the expression of the anti-adhesion molecule CD43 (a marker of immature DC, see Discussion) from $49 \pm 8\%$ positive cells in the control culture to $27 \pm 2\%$ positive cells in the DHEA culture ($P = 0.04$) and $27 \pm 4\%$ positive cells in the DEX culture ($P = 0.04$). Whereas DHEA had no effect on CD40 expression, the addition of DEX to the culture significantly downregulated its expression ($96 \pm 2\%$ positive cells in the control culture vs $52 \pm 7\%$ positive cells in the dexamethasone culture, $P = 0.04$). Both CD43 and CD40 play a role in the further maturation of immature DCs by inducing homotypic interactions (see Discussion) or when ligated by CD40 ligand on T cells respectively.

The expression of CD1a was also downregulated by DEX from $49 \pm 18\%$ positive cells in the control culture to $1 \pm 1\%$ positive cells in the DEX culture (Fig. 1). The presence of DHEA in the culture also resulted in a slight decrease in CD1a expression, from $49 \pm 18\%$ positive cells in the control culture to $32 \pm 0\%$ in the DHEA culture (Fig. 1).

The expression of the mature DC marker CD83 was extremely low in all cultures, underscoring the immature status of all DC populations under study.


(a)
CD80
p=0.05
p=0.05
CD86
CD43
p=0.04
p=0.04
CD40
p=0.04
CD1a
CD83
CD14
p=0.04
100
50
0
control DC
DHEA DC
DEX DC

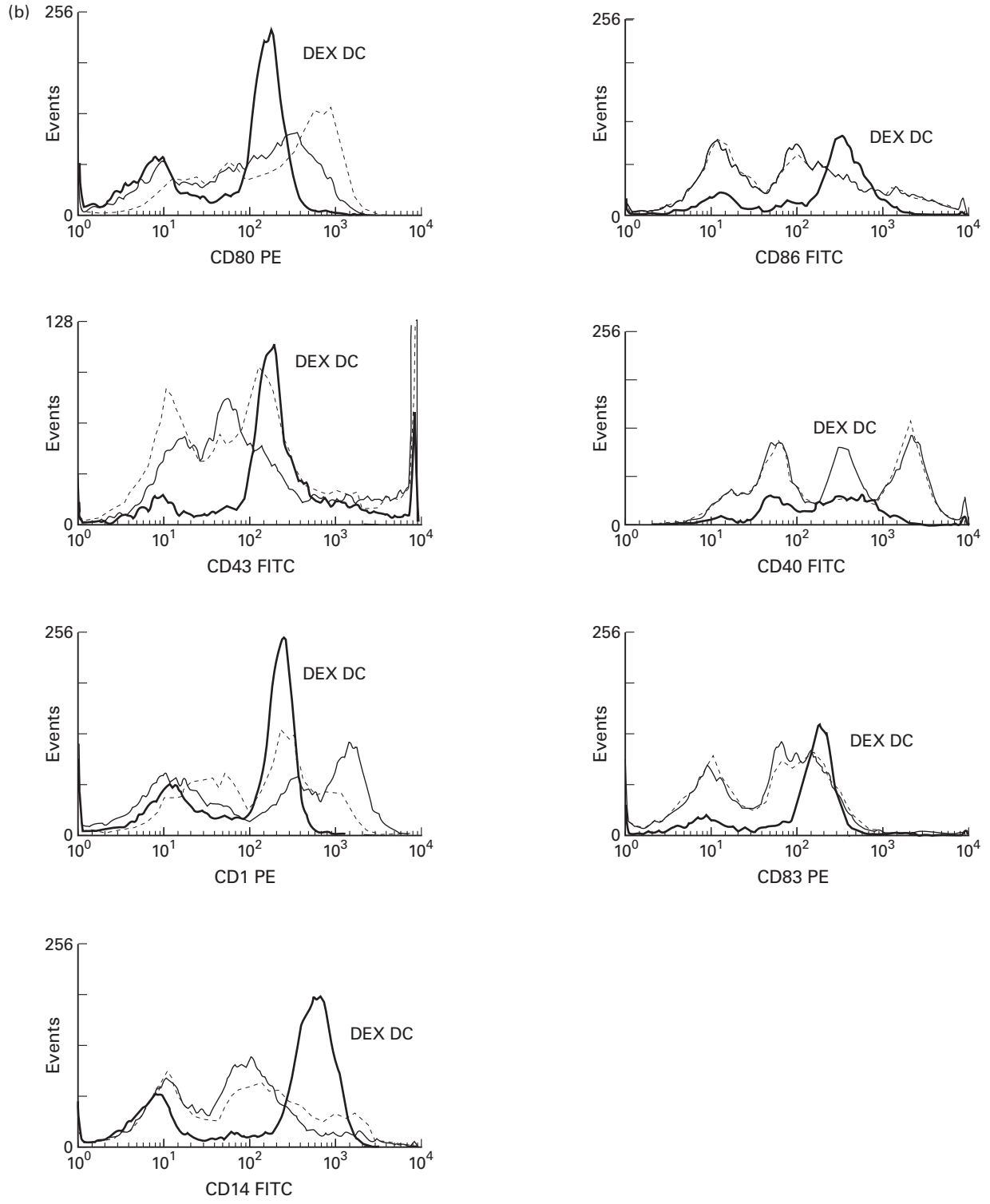


Figure 1 The CD80, CD86, CD43, CD40, CD1a, CD83 and CD14 expression as determined by FACS analysis of control monocyte-derived DC and DC derived from monocytes in the continuous presence of 10^{-6} M DHEA (DHEA DC) or 10^{-6} M dexamethasone (DEX DC). (a) Means (± S.D.) of percentage positive cells are given; only large cells were included. *P* values are as indicated, $n =$ at least five separate experiments. (b) Overlapping histogram profiles of the expression of each marker in a representative experiment. Control DC are represented by a fine line, DHEA DC are represented by a dotted line and DEX DC are represented by a thick line.

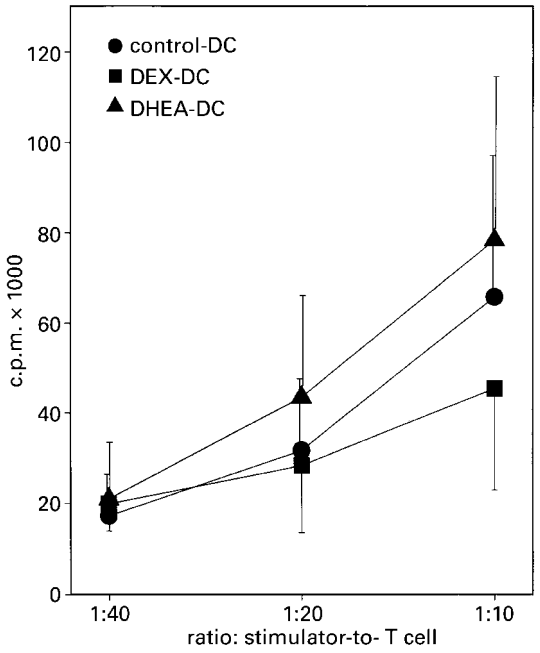


Figure 2 The T cell stimulatory capacity in allo-MLR of the various DC populations studied. Mean c.p.m. × 1000 (± S.D.) are given for various DC:T cell ratios (1:40; 1:20; 1:10).

As expected, most dendritic cells from the control cultures were CD14 negative. A significant increase in CD14 marker expression was seen in cells from cultures containing DEX (74 ± 8% positive cells vs 12 ± 11% positive cells in control cultures, $P = 0.04$). Cells from the cultures containing DHEA did not express CD14 in significantly higher amounts than those from the control cultures. Moreover, staining of cytospin preparations revealed that cells from the DEX cultures were strongly reactive with acid phosphatase in contrast to those from the DHEA- and control cultures which reacted weakly with acid phosphatase.

Thus, in summary, the addition of DHEA enforced the DC-character of the cells with a high CD80 and low CD43 expression, whereas DEX in fact changed the phenotype of the cells into more macrophage-like $CD14^+$ cells, lacking CD1a CD80 and CD40 expression.

Functional differences induced by DHEA and DEX in DC culture

Dendritic cells were co-cultured with allogeneic T cells in mixed leukocyte reactions in order to measure their capacity for stimulating T cell proliferation (Fig. 2). Dendritic cells generated under the influence of DHEA stimulated T cell proliferation as successfully as the control immature dendritic cells. Dendritic/macrophage-like cells generated under the influence of DEX were also able to stimulate T cell proliferation at a comparable level to the control dendritic cells; however, a non-significant difference at a stimulator-to-T cell ratio of 1:10 may suggest that these DC may be slightly inferior at higher ratios. As the number of mixed leukocyte reactions performed was limited ($n = 3$), further experimentation should elucidate whether or not DC generated in the presence of DEX are truly as capable of stimulating T cell proliferation (at all ratios) as control immature DC. The ability of all DC populations generated to stimulate T cell proliferation at a stimulator-to-T cell ratio of 1:40 was comparable to the control PHA response. At a stimulator-to-T cell ratio of 1:10, this response was three times that of the PHA control.

Dendritic cells generated in the presence of DHEA produced IL-10 and IL-12 in not significantly higher amounts than the control DC (Fig. 3). Dendritic/macrophage-like cells generated under the influence of DEX, however, produced significantly more IL-10 than the control DC (1825 ± 503 pg/ml vs. 323 ± 114 pg/ml respectively; mean ± S.E.M.; $P = 0.05$) and virtually shut down their production of IL-12, in contrast to the control DC (0 ± 0 pg/ml vs. 831 ± 490 pg/ml respectively; mean ± S.E.M.; $P = 0.05$).

Discussion

Glucocorticoids are well known for their potent immunosuppressive effects when given in pharmacological doses. There are numerous reports of such suppressive effects on T cells, B cells and macrophages (reviewed in 20, 21). With regard to DC, various studies indicate that exposure to glucocorticoids decreases the function and number of DC *in vivo* (30–32). Literature on the effects of glucocorticoids on the *in vitro* generation of immature and mature DC from their precursors, or on the T cell stimulatory capability of glucocorticoid-exposed DC is increasing (22–26). These reports until now collectively show that the function of DC and the generation of immature and mature DC from monocytes is negatively influenced by the *in vitro* addition of glucocorticoids. Nevertheless, the precise data on the effects of glucocorticoids on DC marker expression, function and maturation are inconsistent. The reasons for the differences among observations are largely unclear, yet it is likely that they include the different maturation states of the DC used and the different exposure times to different doses of glucocorticoids in these experiments. When Van den Heuvel *et al.* (24) exposed GM-CSF/IL-4-cultured human monocytes not continuously, as in this report, but only for brief periods to DEX (early or late in the culture period), a population of immature DC was generated with a decreased accessory capability. This difference in function occurred in these experiments in the absence of changes in the expression of co-stimulatory molecules. Piemonte *et al.* (23) exposed GM-CSF/IL-4-cultured human monocytes not for brief periods, but continuously to DEX; they however used a lower concentration of DEX than in this report (i.e. 10^{-8} M). These authors

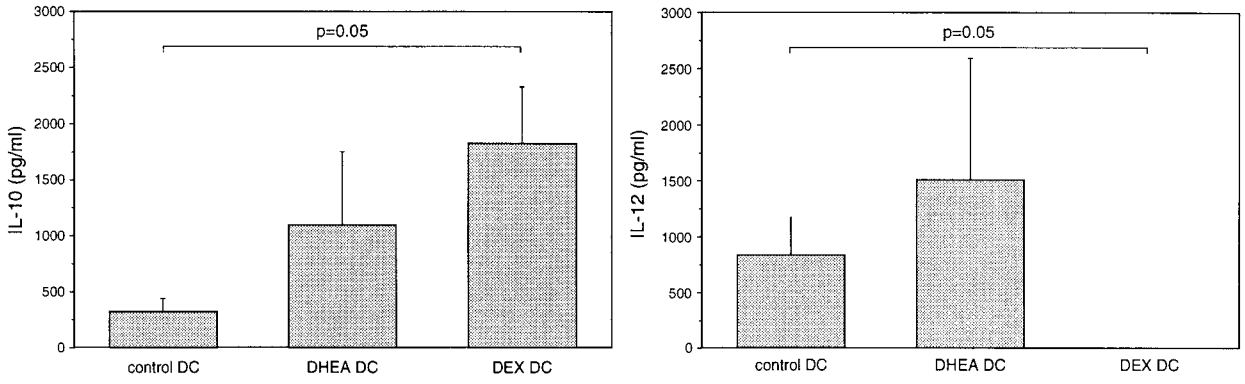


Figure 3 The IL-10 and IL-12 production of control DC, DHEA DC and DEX DC (see Fig. 1 for abbreviations). Means (± S.D.) of production (pg/ml/10^6 cells) are given. *P* values are as indicated, n = at least five separate experiments.

again induced cells with a DC morphology, but with a lower expression of CD86, CD40 and CD1a, a higher expression of MHC-class II, adhesion molecules and CD14, as well as an enhanced antigen uptake via the mannose receptor. The cells again had a poor T cell stimulatory capability. Vieira *et al.* (22) reported that when they similarly produced immature DC in relatively low concentrations of glucocorticoids, they were poor in the production of IL-12 p-70, tumor necrosis factor (TNF)-α and IL-6 (when stimulated with LPS); however, these authors found a normal expression of CD80 and CD86, a normal antigen uptake, and a normal T cell stimulatory capability of such cells.

Vanderheyde *et al.* (25) used already-generated immature monocyte-derived DC and exposed these cells to methyl-prednisolone GM-CSF/IL-4 (100 μg/ml) for 24 h. The cells enhanced their antigen uptake and downregulated their basal expression of CD86 and their T cell stimulation potential. The treatment also prevented LPS-induced maturation, but had limited effects on CD40-induced further maturation. A recent study by Matasic *et al.* (26) also exposed already-generated immature monocyte-derived DC to DEX. The authors confirmed that 500 nM DEX prevented further DC maturation. In fact, the treatment redirected the differentiation of the DC to a more monocyte-macrophage type of cell (high CD14, high CD68, low T cell stimulatory potential).

Here we describe that a continuous exposure of monocytes to a high concentration of DEX (10^{-6} M) completely abolished the generation of cells with an immature phenotype and morphology of DC from monocytes, and induced a set of largely plastic-adherent macrophage-like cells, expressing increased levels of CD14 and decreased levels of 'typical' DC markers such as CD1a, CD40 and CD80. These cells were also strongly reactive to acid phosphatase, yet had a T cell stimulating potential in allo-MLR similar to that of 'immature' DC. They did not, however, produce IL-12 but did produce high quantities of IL-10. The CD80 expression of these macrophage-like cells was very low. In combination with the virtual shutdown of IL-12 production, this suggests that these cells could possibly initiate a Th2-biased response. *In vivo* studies also indicate that exposure to glucocorticoids is able to increase the numbers of (suppressor) macrophages (31, 33). Our data are in line with such an effect, suggesting that the MØ/DC balance is affected by glucocorticoids, which skew this dynamic equilibrium in a macrophage direction when monocytes are continuously exposed to DEX during DC maturation. During this exposure, DEX passively diffuses through the cell membrane, binding to the resting glucocorticoid receptor (GR). The resulting activation of the GR causes it to bind to glucocoticoid-responsive elements (GRE) in the nucleus, influencing gene transcription.

Taken together, a picture is emerging in which glucocorticoids have multiple effects on DC biology. Besides the previously reported negative effects on DC trafficking (34), function (25, 30) and increases in apoptosis of DC (35), glucocorticoids also have shown in many studies variable 'suppressive' and 'altering' effects on the maturation of DC. This probably depends on the dose and time schedule of exposure, and the maturation state of the target DC population.

Serum DHEA-S levels show a steady decline with aging, coinciding with the decline in immune function in old age ('immunosenescence'). When aged individuals, being it experimental animals (36–38) or humans (39) are administered DHEA, their immune function has been claimed to be restored; they become more resistant to infections, their secretion of T cell cytokines (e.g. IL-2) is enhanced, whereas monocyte numbers are increased. Hence, DHEA is viewed by some as an immunostimulating hormone. However, data are also accumulating that such effects of DHEA are minimal or absent (7, 8).

Our data on the effects of DHEA on DC development are in line with the view that DHEA has a modest potentiating effect on immune function. DHEA synergized in our hands with GM-CSF and IL-4 to generate DC with a higher expression of the co-stimulating

molecule CD80 (whereas the expression of CD86 remained equal to that of the control immature DC) and a lower expression of CD43. The sialoglycoprotein CD43 (sialophorin/leukosialin) is a negatively-charged anti-adhesion molecule. When human DC are treated with an antibody to CD43, their clustering capability is enhanced and their phenotypic and functional maturation is mediated (40, 41). Thus, the downregulation of CD 43 expression by DHEA-induced DC reported here may indicate a somewhat greater level of maturation than that of the control DC, which would be in accordance with the higher CD80 expression of these cells. However, CD83 expression was still low after DHEA exposure. An argument against their higher state of maturation is that the DC generated in the continuous presence of DHEA produced IL-10 and IL-12 in similar amounts to the control DC. Their T cell stimulatory capacity was, however, somewhat higher, although this was not statistically significant. Despite this seeming lack of significant functional differences, the phenotypic profile of the DHEA-induced DC (high CD80 expression) could signal the potential of these DC to direct the development of naïve Th cells toward a Th1 phenotype (42). DHEA would then be the first hormone known to possess this capability, which until now has only been demonstrated by pro-inflammatory cytokines such as IL-1 and TNF, and substances like LPS (43).

In saying so, we must be aware that it is still a matter of debate whether immune cells (or any other cells) possess specific receptors for DHEA. The effects of DHEA might not be exerted via DHEA-specific receptors, but rather via receptors for active androgenic metabolites of DHEA-S and DHEA generated in immune cells (44). Specifically leukocytes, including macrophages, possess the sulphatases and other enzymes important in this conversion (45, 46).

In conclusion, the data reported here show that the adrenal hormones DHEA and glucocorticoids both have effects on monocyte-to-immature DC maturation, though in a largely opposite manner. The DC maturation data as presented here are suggestive of DHEA inducing somewhat more mature DC with a possible Th1-skewing potential, whereas glucocorticoids induce macrophage-like APC with a possible Th2-skewing potential. Similar opposing effects of DHEA and cortisol on the Th1-Th2 balance *in vivo* have been suggested (47, 48) and refuted (49, 50) before.

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