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In Vitro Differential Effects of the Antiglucocorticoid RU486 on the Release of Lymphokines from Mitogen-activated Normal Human Lymphocytes

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The synthetic antiglucocorticoid RU486 has multiple effects on the immune system. We have recently reported that RU486 suppresses normal lymphocyte proliferation and downregulates interleukin-2 receptors (IL-2R) by decreasing the accumulation of the β-chain IL-2R mRNA in normal human lymphocytes in culture. To further explore the mechanism of the immunoregulatory actions of RU486, in the present study, we investigated the effects of this molecule on the release of lymphokines from phytohemagglutinin (PHA)-activated normal human peripheral blood lymphocytes (NPBL) in culture. We have found that RU486 differentially regulates the release of lymphokines from PHA-activated NPB lymphocytes. Specifically, RU486 (at concentrations of 1–100 nM) exerts pure antagonist actions by almost completely reversing the inhibitory effects of the glucocorticoid dexamethasone (Dex) on the release of monocyte/macrophages-derived lymphokines, such as IL-1, IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α). Dex decreased in a dose-dependent manner the release of the above four lymphokines, with an ID_{50} of 0.9 ± 0.1, 4.76 ± 0.4, 9.8 ± 1.8, and 1.16 ± 0.2 nM for IL-1, IL-6, IL-8 and TNF-α, respectively. Conversely, RU486 exhibits both agonist and antagonist effects on the release of T-lymphocyte-derived lymphokines. RU486 given alone, exerts agonist/glucocorticoid effects, by decreasing in a dose-dependent manner the release of IL-2 and -3. The maximal inhibitory effect of RU486 was observed at 10 nM and was $64.5 \pm 4.3\%$ of the control value, ($n = 6$, $P < 0.02$) for IL-2 and $59.2 \pm 6.3\%$ ($n = 6$, $P < 0.02$) for IL-3. The ID_{50} of RU486 for the release of IL-2 and -3 were 14.6 ± 2.0 and 11.6 ± 1.9 nM, respectively, i.e. almost similar with those of Dex. Interestingly, when high doses of RU486 (1 μM) were combined with Dex RU486 exhibited antagonist actions by significantly counteracting the inhibitory effects of Dex on IL-2 and -3 release. In conclusion, the antiglucocorticoid RU486 exhibits complex regulatory actions on lymphokine secretion, dependent upon the type of the lymphokine-producing cell. A pure antagonist effect was observed on the release of monocyte-derived IL-1, IL-6, IL-8 and TNF-α. However, when RU486 was given alone it acted as a glucocorticoid agonist on the secretion of T-lymphocyte-derived IL-2 and -3, while combined with the agonist (Dex) it exhibits antagonist effects on the release of the above lymphokines. This molecule still remains unexplored as an immunoregulatory agent. Further studies are needed in order to assess its relevance on pharmacological intervention in the immune system.

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INTRODUCTION

RU486 [17β-hydroxy-11β-(4-dimethylaminophenol) 17α- (prop-1-ynyl) estra-4,9-diene-3-one] binds with high affinity to glucocorticoid and progesterone receptors [1–4]. It has recently been proposed as a contragestive agent, as well as for the pharmacological management of Cushing's syndrome [5, 6]. RU486

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has also been tested, at high doses, for the treatment of advanced and tamoxifen-resistant breast cancer [4, 7, 8]. Recently it was reported that RU486 at high concentrations, (1–10 μM) could suppress lymphocyte thymidine incorporation *in vitro* [9]. These results suggest that at serum concentrations of 1 μM, achieved in women taking the compound as an abortifacient or as an antineoplastic agent [10], RU486 could exert immunosuppressive effects. However, the mechanism of the immunomodulatory actions of this molecule is unknown. Such inhibitory effects of RU486 could be attributed, at least partly, to a decreased expression of interleukin-2 receptors (IL-2R). The IL-2R is expressed in B and T lymphocytes after antigenic or mitogenic stimulation and represents a key molecule in the pathway of the immune response, since it mediates the action of IL-2, a lymphokine known to promote the proliferation of T cells. Indeed, we have recently reported that RU486 downregulates the IL-2R by decreasing the expression of the β chain IL-2R gene in normal human lymphocytes in culture [11].

Another site of action of RU486 as an immunomodulator could be the release of lymphokines which are known to act in a paracrine/autocrine manner as mediators and coordinators of the immune cells. Their most prominent effect is to stimulate proliferation of immune cells, amplifying the initial antigen-specific response. Lymphokines are also involved in general, non-specific defence mechanisms, such as inflammation and immunosurveillance of tumor formation. IL-1, IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α) derive mainly from monocytes/macrophages, whereas IL-2 and -3 are mainly produced by antigen-activated T-lymphocytes [12]. The aim of the present study was to further explore the immunomodulatory actions of RU486. We investigated the effects of RU486 on the release of lymphokines (IL-1, IL-2, IL-3, IL-6, IL-8 and TNF-α) from phytohemagglutinin (PHA)-activated human peripheral blood lymphocytes (NPBL) in culture.

EXPERIMENTAL

Cell cultures

Peripheral blood lymphocytes (PBL) were isolated from freshly obtained heparinized venous blood of healthy volunteers, by Ficoll–Paque density gradient centrifugation. Cells (10^6/ml) were resuspended in complete medium RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Flow Labs, Irvine, Scotland), 2 g/l sodium bicarbonate, 2 mM L-glutamine, 1.5 mg/ml of PHA, 1ng/ml of PMA (Flow Labs), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cell cultures were performed in 5% CO_2/95% air at 37°C in the presence of various concentrations of RU486 or dexamethasone (Dex) (1–1000 nM) and combinations of these reagents. All cell cultures were carried out for 48 h, since at this time the maximal PHA-induced activation of lymphocytes is achieved [13].

Measurement of lymphokines

All culture media for lymphokine determination were stored at −80°C until assayed. The levels of different lymphokines (IL-1, IL-2, IL-3, IL-4, IL-6, IL-8 and TNF-α) in the culture medium were measured by enzyme-linked immunoassays, specific for its lymphokine, using a 'sandwich' method (Quantikine, Rand D Systems, U.S.A.). The results were expressed as pg of the respective lymphokine per mg of total cellular protein, which were determined on whole cellular homogenates by the Bradford method [14] using bovine serum albumin as standard.

Statistical analysis

Following the calculation of the concentration of lymphokines in the culture media (in pg per mg of total cellular protein), the data were finally expressed as percentages of the control, i.e. levels of the corresponding lymphokine in the absence of any steroid. To evaluate the dose–response curve of each steroid and their combination we used two non-parametric statistical methods (Wilcoxon and Kruskal–Wallis) since the levels of lymphokines for its steroid concentration were normalized to the respective controls. Absolute concentration of the respective controls are given in the legend of each figure.

RESULTS

Effects of RU486 on monocyte/macrophage-derived lymphokines

Relative levels of IL-1, IL-6, IL-8 and of TNF-α were measured in the culture medium of blasts activated for 48 h with PHA in the absence and presence of RU486 or Dex at various concentrations (1–1000 nM) and Dex plus 1 μM RU486 (Table 1). Results were expressed as percentage of the control (i.e. cells cultured in the absence of any steroid). As expected, Dex decreased the release of all four lymphokines in a dose-dependent manner. The maximal inhibitory effect of Dex was observed at 10 nM. Specifically, in NPBL cells incubated with 10 nM of Dex, the concentration of these lymphokines in the culture medium was as follows: IL-1, $21 \pm 2\%$ of the control value, (mean $\pm$ SEM, $n = 6$, $P < 0.001$) [Fig. 1(A)]; IL-6, $49.6 \pm 3.1\%$ ($n = 6$, $P < 0.03$); IL-8: $54.9 \pm 4\%$ ($n = 6$, $P < 0.03$) (Fig. 3); and TNF-α, $60.3 \pm 3.7\%$ ($n = 6$, $P < 0.04$) (Fig. 4). RU486 given alone at concentrations of 1–100 nM did not show a statistically significant effect on the release of these lymphokines. However, RU486 at 1 μM almost completely reversed the inhibitory effect of Dex on IL-1 [Fig. 1(A)], IL-6 and IL-8 (Fig. 3) and TNF-α (Fig. 4).

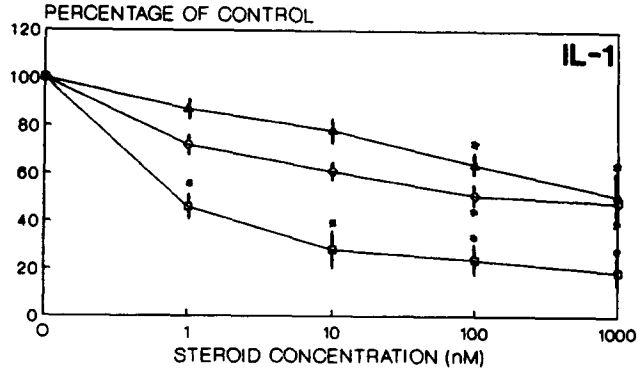
Table 1. Effect of RU486 on the release of lymphokines from NPB lymphocytes in vitro

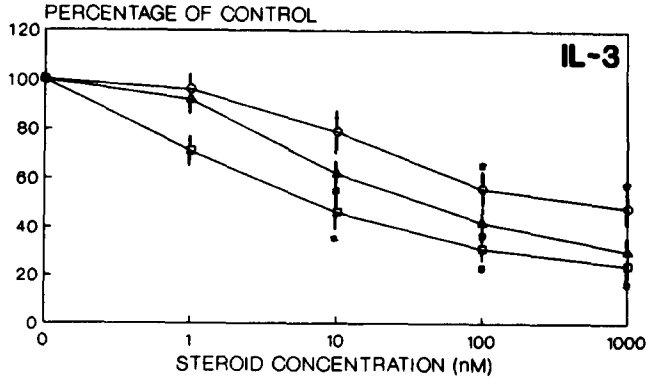
Steriod	IL-1	IL-2	IL-3	IL-4	TNF-α	IFN-γ
Dex	⇓⇓⇓ 0.9 ± 0.1*	⇓⇓⇓ 12.9 ± 2.1	⇓⇓⇓ 3.8 ± 1.2	NE	⇓⇓⇓ 9.8 ± 1.8	⇓⇓⇓ 1.16 ± 0.2
RU486	NE	⇓⇓⇓ (agonist) 14.6 ± 2	⇓⇓⇓ (agonist) 11.6 ± 1.9	NE	⇓	NE
Dex + RU486	⇓ (antagonist)				NE (antagonist)	⇓ (antagonist)

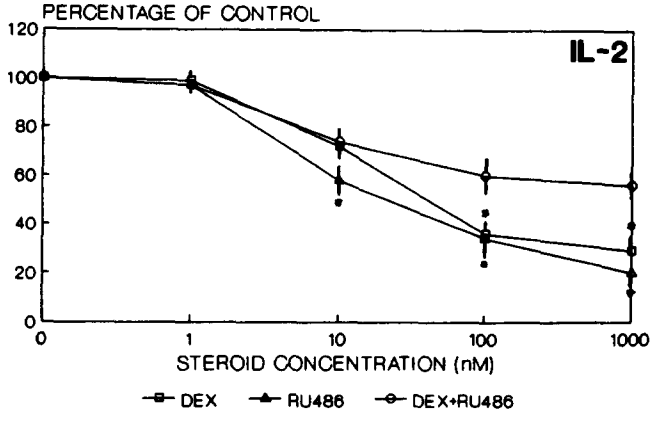
NE, no significant effect; ⇓, decrease; *ID_{50} in nM $\pm$SEM, $n = 6$.

Effects of RU486 on T-lymphocyte-derived lymphokines

NPB lymphocytes were activated for 48 h with PHA in the absence and presence of RU486 or Dex at various concentrations (1–1000 nM), then the levels of IL-2 and -3 were measured in the culture media. The results were expressed as percentage of the control (i.e. cells cultured in the absence of any steroid). Dex decreased the release of IL-2 and -3, in a dose-dependent manner [Figs 1(B) and 2(A), respectively]. The maximal inhibitory effect of Dex was observed at 100 nM, showing an ID_{50} for the release of IL-2 and -3 of 12.9 ± 2.1 and 3.8 ± 1.2 nM, respectively. RU486 exhibited agonist effects on monocyte-derived IL-1, IL-6, IL-8 and TNF-α release. The maximal inhibitory effect of RU486 was observed at 10 nM and was $64.5 \pm 4.3\%$ of the control value for IL-2 (mean $\pm$ SEM, $n = 6$, $P < 0.02$) [Fig. 1(B)] and $59.2 \pm 6.3\%$ for IL-3 ($n = 6$, $P < 0.02$) [Fig. 2(A)]. The ID_{50} of RU486 was 14.6 ± 2.0 and 11.6 ± 1.9 nM for the release of IL-2 and -3, respectively, i.e. similar to those of Dex.







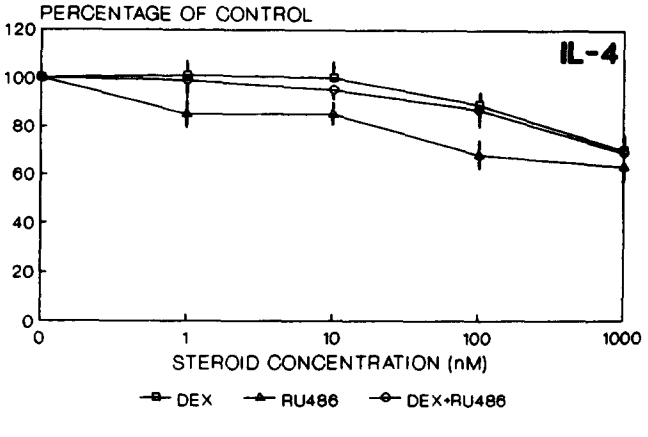


Fig. 1. Effect of RU486 on the release of IL-1 and -2. NPB lymphocytes were incubated for 48 h with PHA in the absence and presence of various concentrations of RU486 (△), Dex (□) and Dex plus 1 μM RU486 (○). Results are expressed as percentage of control (mean ± SEM, $n = 6$). The absolute values of control for IL-1 and -2 were 212 ± 12 and 816 ± 34 pg/mg of protein, respectively. *$P < 0.05$.

Fig. 2. Effect of RU486 on the release of IL-3 and -4. NPB lymphocytes were incubated for 48 h with PHA in the absence and presence of various concentrations of RU486 (△), Dex (□) and Dex plus 1 μM RU486 (○). Results are expressed as percentage of control (mean ± SEM, $n = 6$). The absolute values of control for IL-3 and -4 were 121 ± 11 and 89 ± 3 pg/mg of protein, respectively. *$P < 0.05$.

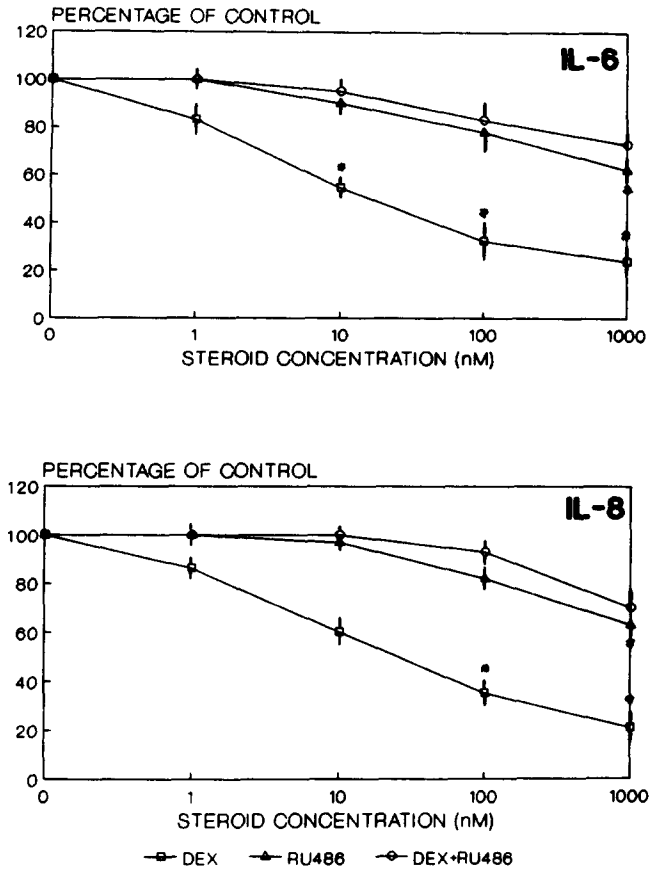


Fig. 3. Effect of RU486 on the release of IL-6 and -8. NPB lymphocytes were incubated for 48 h with PHA in the absence and presence of various concentrations of RU486 (△), Dex (□) and Dex plus 1 μM RU486 (○). Results are expressed as percentage of control (mean ± SEM, $n = 6$). Absolute values of control for IL-6 and -8 were 21 ± 11 and 89 ± 3 pg/mg of protein, respectively. *$P < 0.05$.

However, when RU486 (1 μM) was combined with Dex (1–1000nM), it significantly reversed the inhibitory effects of the latter on the release of IL-2 and -3, thus exerting antagonist effects [Figs 1(B) and 2(A), respectively].

DISCUSSION

Our data show that RU486 has multiple effects on PHA-activated human lymphocytes. Specifically it appears that RU486 (a) exerts pure antagonist actions on the release of monocyte-derived lymphokines IL-1, IL-6, IL-8 and TNF-α by significantly reversing the inhibitory effects of Dex and (b) when given alone exhibits agonist-glucocorticoid effects by decreasing the release of T-lymphocyte-derived lymphokines IL-2 and -3, while combined with Dex acts as an antagonist.

It is known that glucocorticoids have multiple and complex effects on the immune system. Most of these effects appear to be mediated by the inhibition of the synthesis and release of several lymphokines, including IL-1 [15–18], IL-2 [19], IL-3 [20], IL-6, IL-8 [21, 22] and TNF-α [23]. Our data confirm these observations and also show that the above inhibitory effects of glucocorticoids can be counteracted by the antagonist RU486. However, this molecule possess glucocorticoid-like agonist properties on the release of IL-2 and -3. These observations further demonstrate the agonist activity of RU486 and could be explained by the high affinity of its binding to glucocorticoid receptors. The possibility that RU486 has agonist effects on glucocorticoid receptors has been addressed recently. Indeed several studies have demonstrated the weak glucocorticoid activity of RU486 in the hypothalamo–pituitary axis *in vitro* and *in vivo* [3, 6]. Our findings support these agonist actions of RU486 in the immune system, suggesting that this compound at high concentrations may display immunosuppressive effects. However, we have found that when lymphocytes were exposed to a combination of Dex plus RU486, the latter almost completely prevented the inhibitory effect of the former, thus acting as an antagonist. This intriguing effect of RU486 in the presense of the agonist has already been described in another system. Indeed, in human endometrium RU486 exerts agonist effects on DNA polymerase activity, thus mimicking the inhibitory effects of progesterone, then when it is combined with progesterone it reverses the effects of the latter, acting as an antagonist [3]. The mechanism of this phenomenon remains unclear. However, a possible explanation is that homodimers of glucocorticoid receptors (GR) (or progesterone receptors) which are composed of one molecule of the GR–agonist complex and one molecule of the GR–antagonist complex behave differently than homodimers which are 'homogenous', i.e. contain two molecules of GR bound exclusively to the agonist or antagonist. It is interesting to note that in the case of progesterone receptors (PR), homodimers which contain one molecule of PR–agonist and one molecule of PR–RU486 have lower affinity for the DNA [16].

The finding that RU486 clearly exerts agonist actions in T-lymphocytes while in monocytes/macrophages these actions are minimal or absent suggests that there is a cell-specific action of RU486 on immune cells. The mechanisms on which these differences depend are unclear. For instance, there is not enough information on the properties of glucocorticoid receptors in different immune cell types. On the other hand, factors interfering with RU486 regulation of lymphokine gene expression might at present be dependent upon immune cell type. Indeed, it has recently been reported that in immature murine lymphocytes, RU486 acted as a glucocorticoid agonist only in T-cells with highly active protein kinase A activity, while in the absence of this enzyme RU486 exhibits antagonist actions [24]. Additionally, in murine T-cells, RU486 in the presence of cAMP exerts agonist actions on cell apoptosis by a mechanism independent of receptor transformation, most probably by promoting an interaction between GR and other cAMP-dependent gene-specific regulatory proteins [24]. Moreover, it has been shown that the

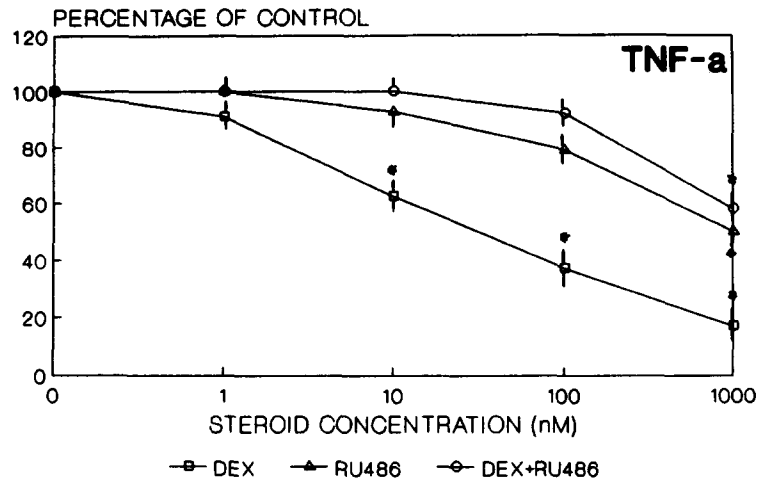


Fig. 4. Effect of RU486 on the release of TNF-α. NPB lymphocytes were incubated for 48 h with PHA in the absence and presence of various concentrations of RU486 (△) and Dex (□). Results are expressed as percentage of control (mean ± SEM, $n = 6$). Absolute values of control for TNF-α were 81 ± 4 pg/mg of protein. *$P < 0.05$.

immunosuppressor FK506 inhibits the antagonist actions of RU486 on mouse fibroblasts in culture [25]. FK506 is specifically recognized by heat shock protein p59 which seems to be a component of GR through its binding to hsp90 [26, 27]. It is possible that regulatory factors of glucocorticoid actions, such as p59, may be present in a specific immune cell type, while missing on others, thus explaining the differences between monocytes/macrophages and T-cells.

Recently, a rich network of uterine lymphokines, such as IL-1, interferon-γ, TNF-α, granulocyte-macrophage colony stimulating factor, and colony stimulating factor have been described. These lymphokines are mainly synthesized from endometrial stromal or epithelial cells and they affect blastocyst attachment and implantation, trophoblast outgrowth or menstruation [28, 29]. Thus, it is plausible to think that the regulatory effects of RU486 on lymphokine secretion could also take place within the uterine cavity and that at least part of the action of RU486 on human endometrium might be due to its interference with the synthesis or secretion of uterine lymphokines. In addition, the antiproliferative effects of RU486 in hormone-resistant breast cancer cells might be partly attributed to its possible local immunoregulatory effects at the tumor level.

In conclusion, the antiglucocorticoid RU486 exhibits complex immunoregulatory actions which can be attributed to its regulatory effects on lymphokine secretion as well as on lymphokine receptor expression. This molecule still remains unexplored as an immunomodulatory agent. Further studies are needed in order to assess its relevance in the pharmacological intervention of the immune system, for example its usefulness in specifically reversing some of the immunoregulatory effects of glucocorticoids.

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