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THE ANTIGLUCOCORTICOID RU486 DOWNREGULATES THE EXPRESSION OF INTERLEUKIN-2 RECEPTORS IN NORMAL HUMAN LYMPHOCYTES

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Summary—The effects of the antiglucocorticoid RU486 on the expression of low and high affinity interleukin-2 receptors (IL-2R) in phytohaemagglutinin (PHA)-activated human peripheral blood lymphocytes were investigated. We demonstrated that RU486 inhibits in a dose-dependent way the expression of both classes of IL-2R, thereby mimicking the effects of the glucocorticoid agonist dexamethasone. The maximal effect on the low affinity binding sites was observed at 10 μM (28 $\pm$ 2% of control, $P < 0.001$) and on the high affinity IL-2R at 1 μM (from 2938 $\pm$ 74 to 437 $\pm$ 108 binding sites per cell, $P < 0.001$). This inhibition of IL-2R expression occurs at a pretranslational level since RU486 decreased the accumulation of β-chain IL-2R mRNA transcripts. Our data support the concept that the antiglucocorticoid RU486 at pharmacological concentrations can exert agonistic-immunosuppressive effects.

INTRODUCTION

RU486 [17β-hydroxy-11β-(4-dimethylamino-phenol) 17α-(prop-1-ynyl)estra-4,9-diene-3-one] binds with high affinity to glucocorticoid and progesterone receptors, exerting both antagonistic and agonistic effects [1–3]. It is clinically used as an abortifacient agent and it has also been tested successfully in the management of Cushing's syndrome [4, 5]. Recently it was reported that RU486 at high concentrations, (1–10 μM) could suppress lymphocytes thymidine incorporation *in vitro* [6]. These results suggest that at serum concentrations of 1 μM, achieved in women taking the compound as an abortifacient [7], RU486 could exert immunosuppressive effects. Such inhibitory effects of RU486 could be attributed 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 interleukin-2, a lymphokine known to promote the proliferation of T-cells [8]. Lymphocytes possess mainly two classes of IL-2 binding sites with different affinities but a shared subunit named α-chain or Tac molecule. The low affinity binding site (K_d: 10 nM) corresponds to the α-chain of IL-2R which is a 55 kDa glycoprotein [9]. The high affinity binding site (K_d: 10 pM) consists of the α-chain non-covalently linked to the β-chain, a 75 kDa protein [10, 11]. Recently it was shown that glucocorticoids inhibit the expression of IL-2R in human lymphocytes *in vitro* [12] thus inhibiting lymphocytes proliferation.

The purpose of our study was to further investigate the actions of RU486 on immune functions. More precisely we focused on the effects of this compound on the expression of interleukin-2 receptors in phytohaemagglutinin-activated normal human peripheral blood lymphocytes (PHA-blasts).

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, U.K.), 2 g/l sodium bicarbonate, 2 mM L-glutamine, 1.5 μg/ml of PHA, 1 ng/ml of PMA

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(Flow Labs, Irvine, U.K.), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cell cultures were performed in 5% CO_2/95% air at 37°C in the presence of various concentrations of RU486 or dexamethasone (10^{-5}–10^{-9} M) and combinations of these reagents. All cell cultures were carried out for 48 h, since at this time the maximal PHA-induced IL-2R expression is achieved [13].

Thymidine and methionine incorporation assay

Thymidine and methionine incorporation in PHA blasts was determined essentially as described [13]. Briefly 0.2 ml of final cell suspension were added to flat-bottomed microtiter cultures and were incubated at 37°C in 5% CO_2/95% air for 48 h in the absence and presence of various steroids and their combinations. At the end of the incubation each well was pulsed with 1 μCi of [methyl-^{3}H]thymidine (sp. act.: 20 Ci/mmol, New England Nuclear, Boston, Mass.) for 24 h, or with 1 μCi of [^{35}S]methionine (sp. act.: 400 Ci/mmol, New England Nuclear, Boston, Mass.) for 4 h. Cell-associated radioactivity was determined by liquid scintillation.

Indirect immunofluorescence for the α-chain of IL-2 receptor

An indirect immunofluorescence assay was performed as described in detail [14, 15] by employing the anti-Tac (IgG 2α) monoclonal antibody ($10T_{14}$, Immunotech, France). This antibody recognizes the α-chain of IL-2R. The retained anti-Tac antibody was stained with FITC (Immunotech, France) and the cells positive for the α-chain were counted by fluorescence microscopy. At least 300 viable cells were counted for each experimental point.

Radiolabeled IL-2 binding assay

Initially, PHA-activated blasts were pre-incubated (for 1 h at 37°C) in fresh culture medium, then they were washed three times with RPMI medium to remove endogeneous IL-2, bound to IL-2R. Approximately 5×10^5 cells were incubated with serial concentrations of pure recombinant [^{125}I]IL-2 (sp. act.: 600 Ci/mmol, New England Nuclear, Boston, Mass.) ranging from 10 to 700 pM. The cells were incubated with [^{125}I]IL-2 in 5% CO_2/95% air for 1 h at 37°C. These conditions allow for optimal saturation of high affinity binding sites [16]. At the end of the incubation 150 μl of cell suspension were overlayed on a microtube containing 200 μl of binding oil (phthalic acid-bir (3,5,5-trimethyl-hexyl) ester and dibutyl–phthalate 1:1.5 Sigma, St Louis, Mo.) and centrifuged at 2000 *g* for 30 min. The pellets containing the bound radioactivity, were counted in a LKB-Wallac automatic gamma counter. The number of binding sites per cell and the apparent dissociation constants (K_d) were determined by using the LIGAND computer program of Munson and Rodbard [17].

RNA blot analysis

Total RNA of PHA blasts was extracted with hot phenol. After size-fractionating RNA (10 μg/lane) by electrophoresis through 1% agarose gels containing 6% formaldehyde and 2 μg/dl of ethidium bromide, gels were viewed under u.v. irradiation to assess the integrity of the RNA and to verify that equal amounts of RNA had been loaded in all wells. After transfer of RNA to Gene Screen nylon membranes (New England Nuclear, Boston, Mass.) the filters were prehybridized and hybridized with cDNA probes, radioactively ^{32}P-labeled by random priming, using oligodeoxynucleotides, (sp. act. 5×10^7 cpm/μg) essentially according to Feinberg and Vogelstein [18]. Hybridizations were carried out as described [19]. Autoradiography using Kodak XR film occurred at -80°C in the presence of an intensifying screen. The human cDNA probe for the detection of the mRNAs of the IL-2 receptor β-chain was a 1.05 kb SacI, BamH1 fragment inserted into α pUC19 plasmid [10]. For control hybridizations, the RNA blots were rehybridized with a rat β-actin cDNA Pst1 fragment inserted into a pUC18 plasmid [20].

RESULTS

Thymidine incorporation

The effect of RU486 on [methyl-^{3}H]thymidine ([^{3}H]Thd) incorporation into PHA-activated blasts and its influence on the suppressive effects of dexamethasone (DEX, a synthetic glucocorticoid) were studied. RU486 at 10, 1 and 0.1 μM significantly decreased the incorporation of [^{3}H]Thd by 33, 34 and 43% of controls, respectively, ($P < 0.02$). DEX also inhibited lymphocyte [^{3}H]Thd incorporation in a dose-dependent fashion at concentrations from 10 to 1000 nM (Fig. 1). Interestingly, RU486 at 1 μM significantly reversed the DEX-induced suppression of lymphocyte thymidine incorporation. These

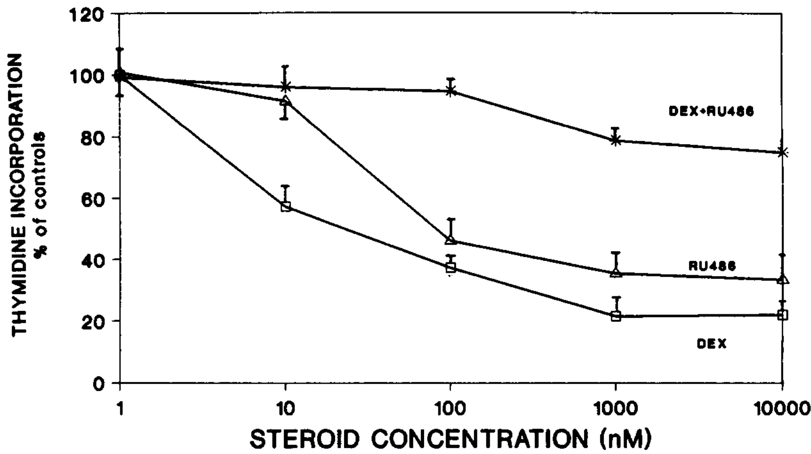

Fig. 1. Effects of RU486 on thymidine incorporation. PHA blasts were incubated for 48 h with various concentrations of RU486 (△), DEX (□) and RU486 plus 1 μM DEX (×) then pulsed for 18 h with 1 μCi of [^{3}H]thymidine. Results were expressed as percentage of control (mean ± SEM of 3 experiments). Absolute value of control: 123.000 ± 10.942 cpm per 5×10^5 cells.

results are in agreement with recently published data [6], although we observed that RU486 even at 0.1 μM exerted an important suppressive effect on [^{3}H]Thd incorporation.

To rule out a cytotoxic effect of steroids application, cell viability was estimated by performing cell counts in the presence of trypan blue and measuring [^{35}S]methionine incorporation as described in Experimental. In all treatments lymphocyte viability was greater than 90% and no significant reduction of [^{35}S]methionine incorporation was observed (data not shown).

RU486 inhibits the expression of the α-chain of IL-2 receptor

Relative levels of α-chain IL-2R expression were measured by immunofluorescence assay on PHA-stimulated (for 48 h) blasts in the absence and presence of RU486 and/or DEX at various concentrations (1 nM–10 μM). As shown in Figs 2 and 3, RU486 at 10, 1 and 0.1 μM significantly decreased the expression of the α-chain at 28, 71 and 79% of control, respectively, ($P < 0.05$). In addition, DEX at 10, 1 and 0.1 μM caused an inhibition of

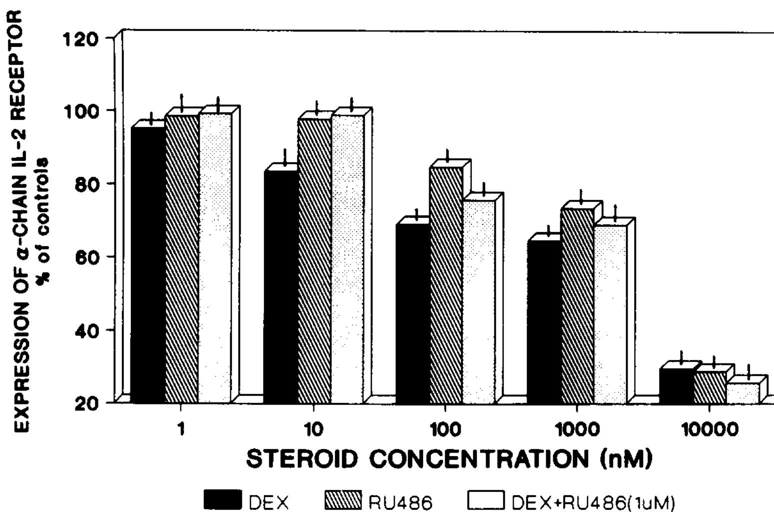

Fig. 2. Effects of RU486 on the expression of the α-chain of IL-2 receptors. PHA blasts were incubated for 48 h with various concentrations of RU486, DEX and RU486 plus 1 μM DEX. α-chain positive cells were recognized by anti-Tac monoclonal antibodies, stained with FITC and counted with fluorescence microscopy. Results were expressed as percentage of control (mean ± SEM of 3 experiments). Absolute value of control: 247 ± 12 out of 300 counted cells, found α-chain positive.

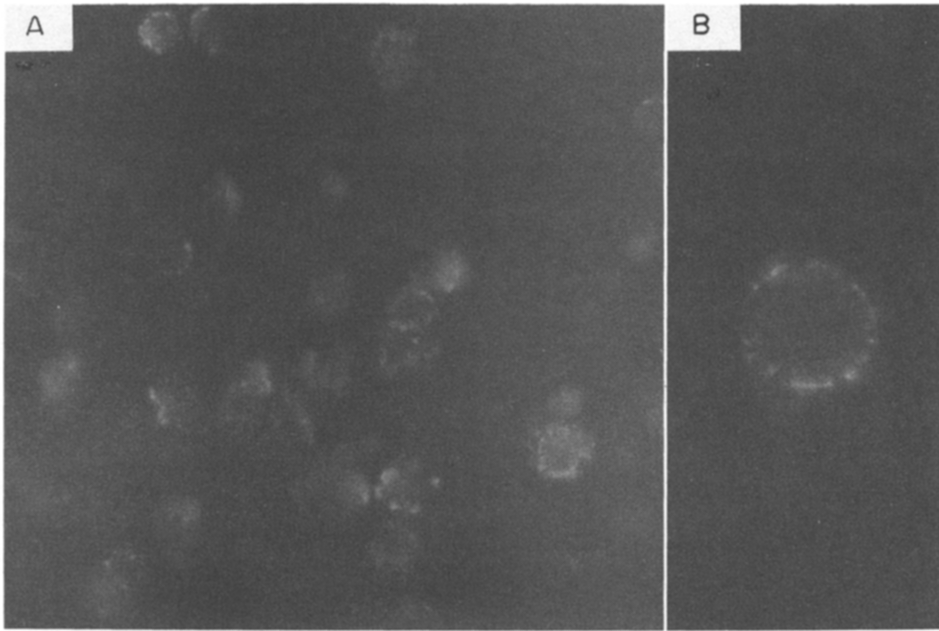

Fig. 3. Indirect immunofluorescence of α-chain positive PHA blasts. Peripheral blood lymphocytes were cultured for 48 h with 1.5 μg/ml of PHA then incubated with an anti-Tac monoclonal antibody. The retained anti-Tac antibody was stained with FITC. Magnification: (A) ×400, (B) ×1000.

the expression of the α-chain IL-2 receptor (23, 64 and 69% of controls, respectively, $P < 0.05$) confirming recently published data [12]. No significant reversal of DEX induced suppression of the expression was demonstrated with 1 μM of RU486. On the contrary their combination diminished α-chain expression at least as much as either steroids tested separately.

RU486 inhibits the expression of high affinity IL-2 receptor

Because anti-Tac (anti α-chain) antibody does not distinguish between high and low affinity forms of IL-2R [15], we also determined the effects of RU486 on the expression of high affinity IL-2R with the use of a quantitative binding assay. Figure 4 compares the number of

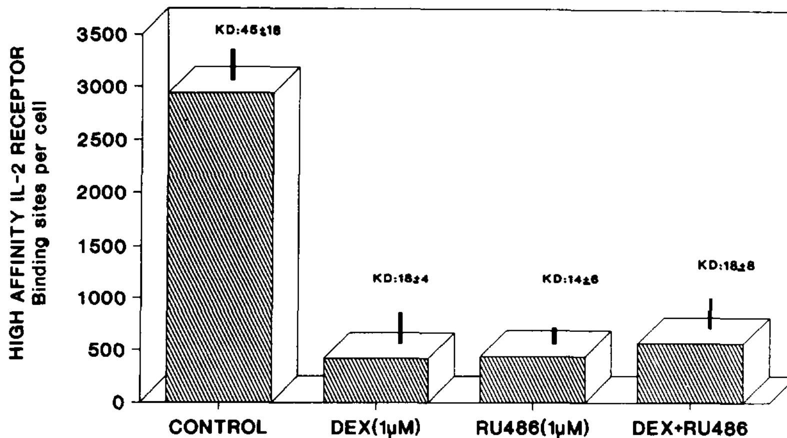

Fig. 4. Effects of RU486 on the expression of high affinity IL-2 receptors. PHA blasts were incubated for 48 h with RU486 (1 μM), DEX (1 μM) and their combination, then were incubated for 1 h at 37°C with various concentrations of recombinant [^{125}I]interleukin-2 (10–700 pM). Bound radioactivity was isolated with binding oil centrifugation and the number of binding sites was determined by Scatchard analysis. Results were expressed as number of binding sites per cell (mean ± SEM of 3 experiments) K_d: dissociation constant.

Table 1. Effects of RU486 on the expression of high affinity IL-2 receptor in PHA-activated lymphocytes

RU486 (nM)	Number of binding sites per cell	K_d (pM)
0	2963	45
1	1832	18
10	1623	35
100	1106	15
1000	448	11

Freshly isolated human normal lymphocytes were activated with PHA for 48 h in presence of various concentrations of RU486 then they were incubated for 1 h at 37°C with various concentrations of [^{125}I]interleukin 2 (10–70 pM). The number of binding sites per cell and the K_d were determined by Scatchard analysis.

high affinity binding sites of IL-2R per cell. As shown, RU486 at 1 μM significantly diminished the high affinity IL-2R from 2938 ± 74 to 437 ± 108 binding sites per cell, ($P < 0.001$) thus mimicking the suppressive effect observed by DEX. The above inhibitory effect of RU486 was shown to be dose-dependent (Table 1, Fig. 5). The combination of RU486 with DEX (both at 1 μM), resulted also in a significant suppression of the expression of high affinity binding sites, from 2938 ± 74 to 565 ± 23, ($P < 0.001$).

RNA blotting

In an effort to delineate the mechanism whereby RU486 suppresses the PHA-induced expression of IL-2R, we investigated the effects of this inhibitory agent on the accumulation of mRNA for the β-chain of IL-2R by standard RNA blotting techniques.

We have shown that control cultures of PHA blasts (absence of any steroid application) expressed a 4 kb mRNA transcript, recently described as the β-chain IL-2R mRNA [10] (Fig. 6). However exposure of PHA blasts for 8 h to 1 μM of RU486 or DEX resulted in a

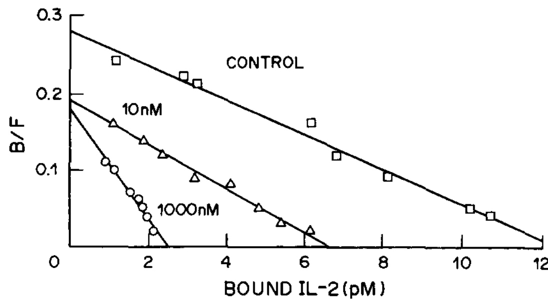

Fig. 5. Specific binding of [^{125}I]IL2 to PHA blasts exposed to RU486. PHA blasts were cultured for 48 h in absence (□) and in presence of RU486 at 10 nm (△) and 1000 nM (0), then they were incubated for 1 h at 37°C with various concentrations of [^{125}I]IL-2 (10–700 pM). Bound radioactivity was isolated with binding oil centrifugation. Data are expressed as Scatchard plots (B/F: Bound IL-2/Free IL-2).

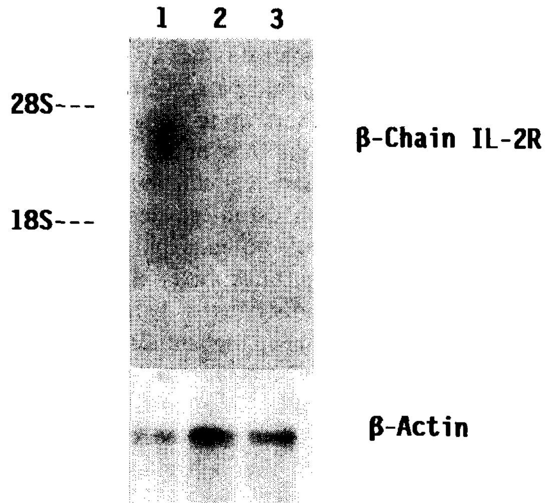

Fig. 6. Effects of RU486 on the accumulation of IL-2 receptor β-chain mRNA. PHA blasts were incubated for 8 h with RU486 (1 μM) or DEX (1 μM), then total RNA was isolated and hybridized with radiolabeled cDNA probes for the β-chain of IL-2R as described in Experimental. For control hybridizations RNA blots were rehybridized with a rat β-actin cDNA. Lanes 1: control. 2: RU486, 3: DEX.

highly significant diminution of blasts content of β-chain IL-R mRNA. As shown in Fig. 4, no signal for the β-chain mRNA was detected under those experimental conditions, a finding that is in full agreement with the highly significant effect of both steroids on the high affinity IL-2R, as shown in Fig. 4.

DISCUSSION

The results presented here clearly demonstrate that the glucocorticoid antagonist RU486 exerts an inhibitory effect on mitogen-induced T-cell proliferation, which is associated with (a) decreased membrane expression of the corresponding α-chain of IL-2R and diminished number of high affinity IL-2-binding sites, and (b) decreased accumulation of IL-2R β-chain mRNA transcripts.

Recently, it was reported that glucocorticoids inhibit the expression of both high and low affinity IL-2R on PHA-activated cells which is due to an impaired accumulation of IL-2R α-chain mRNA. In this particular case, the decreased number of high affinity IL-2R per cell could explain the glucocorticoid-mediated inhibition of cell proliferation [12]. In the present study we observed that RU486, which is a potent competitor of dexamethasone at the level of glucocorticoid receptor, mediated a similar effect on both mitogen-induced cell accumulation of IL-2R β-chain mRNAs and membrane

expression of IL-2R α-chain. These observations further demonstrate the agonistic activity of RU486 and could be explained by the high affinity of its binding to glucocorticoid receptors. The possibility of agonistic activity of RU486 on glucocorticoid effects has recently been explored. Several studies have demonstrated weak glucocorticoid activity for RU486 in the hypothalamo–pituitary axis *in vitro* and *in vivo* [2, 21]. Our findings extend those agonistic actions of RU486 at the immune system, suggesting that this compound at high concentrations may display immunosuppressive effects.

The evaluation of the antiglucocorticoid effect of RU486 revealed an heterogeneous pattern. Indeed, RU486 could reverse the dexamethasone-induced impaired cell proliferation (Fig. 1) whereas no similar effects could be observed on membrane expression of high affinity IL-2R (Fig. 4). Since mitogen-induced proliferation of peripheral blood lymphocytes (PBL) depends on the binding of IL-2 to high affinity IL-2R and subsequent internalization of the complex [11], the above observations seem to indicate that production of IL-2 by activated cells and expression of IL-2R α-chain display a differential sensitivity to the action of RU486. Experiments are in progress in our laboratory to further investigate this point.

As already anticipated, our findings also showed that RU486, at high concentrations (10–0.1 μM), suppressed lymphocyte proliferation as assessed by thymidine incorporation, thus confirming recently published data [6]. Interestingly under the same experimental conditions, RU486 did not modify neither methionine incorporation nor cell viability. Those findings considered together indicate that the inhibitory effects of RU486 should not be attributed to a cytotoxic effect and insinuate that this compound may provoke a resting of lymphocyte proliferation.

In conclusion, our data support the concept that antiglucocorticoid RU486 at pharmacological concentrations can exert agonistic-immunosuppressive effects by decreasing the expression of low and high affinity IL-2R in human lymphocytes *in vitro*. Immunosuppression could possibly occur at clinically used doses, although other variables present *in vivo* could alter this effect.

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