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Effect of RU 38486 on TNF production and toxicity

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Glucocorticoid steroids provide considerable protection against the systemic toxicity of tumor necrosis factor-α (TNF-α, cachexin). In animal experiments RU 38486 (mifepristone), a steroid antagonist, increased the synthesis of TNF and sensitized the animals to the cytotoxic action of TNF. As compared to the control and methylprednisolone-treated groups, mifepristone significantly increased the level of TNF in the serum, liver and spleen of lipopolysaccharide (LPS)-treated animals. In tissue cultures RU 38486 induced the TNF synthesis of myeloid cells and increased the TNF production of genetically modified HeLa cells, which synthesize TNF constitutively. Normal and tumor cell cultures exhibited increased sensitivity toward TNF in the presence of mifepristone.

Tumor necrosis factor-α; RU 38486; Mifepristone; Endotoxin; Glucocorticoid

1. INTRODUCTION

Tumor necrosis factor (TNF) shows a very high specificity in its cytotoxicity towards certain tumor cells [1]. At the same time, TNF is a lymphokine with powerful immunostimulating effects and causes severe systemic consequences at elevated levels [2]. Furthermore, TNF [3,4] and other cytokines [5] have been also implicated in the pathogenesis of septic shock, which is one of the most common causes of death in intensive care units today.

Despite advances in technology and in antibiotic therapy, the mortality rate for septic shock remains in excess of 50% [6,7]. Glucocorticoid hormones are known to protect experimental animals very effectively against the lethal effect of bacterial endotoxins [8], but the beneficial influence of these hormones in clinical and experimental forms of septic shock remains a question of debate [9–11]. Antagonists of steroid hormones provide a new framework of action not only because they are of potential clinical use, but more so because they permit molecular dissection of hormone-dependent processes.

The antiglucocorticoid action of mifepristone, or RU 38486, has been reviewed elsewhere [12,13]. In earlier studies, we have shown that this glucocorticoid antagonist sensitizes both Swiss albino OF1 and endotoxin low-responder, C3H/HeJ mice to endotoxin lethality [14]. We have also shown that RU 38486 sensitizes endotoxin-tolerant (endotoxin-pretreated) and normal mice to the lethal effect of septic and endotoxin shock [15,16].

Tumor necrosis factor-α is the primary mediator of the pathogenesis of endotoxin and septic shock [17,18]. Glucocorticoids inhibit LPS-induced TNF production [19] and TNF can stimulate pituitary adrenocorticotropin secretion, resulting in the release of corticosterone [20,21]. All these findings suggest an important role of the neuroendocrine axis in the secretion of steroid hormones and in the modulation of cytokine production. On this basis, we set out to study the influence of RU 38486 on TNF production and toxicity.

2. MATERIALS AND METHODS

2.1. *Animals*

Male NMRI mice (LATI Animal House, Gödöllő, Hungary), 30–35 g in weight, were housed with free access to pellet food and water at all times.

2.2. *Chemicals*

Mifepristone, RU 38486, was from Roussel-Uclaf, France. It was incorporated into phospholipid liposomes. Multilamellar liposomes were prepared from phosphatidylcholine (Sigma) and cholesterol (Sigma) according to the method described earlier [22].

Methylprednisolone (Orion, Finland) and RU 38486 were injected intravenously just before the lipopolysaccharide (LPS) treatment. *Escherichia coli* 026:B6 lipopolysaccharide (Difco, lot no. 672736) was suspended in isotonic, nonpyrogenic saline.

2.3. *Cloning and production of tumor necrosis factor*

TNF-α (human tumor necrosis factor-α, cachexin) gene was isolated from a human gene library. The *Msp*I-*Eco*RI fragment, coding for approx. 80% of the fourth exon of the TNF gene was ligated with a 102 bp synthetic oligonucleotide coding for the missing N-terminal part of the mature protein. The resulting construct was cloned into the *Bam*HI site of the expression vector pDR540 (Pharmacia Fine Chemicals).

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E. coli cells harboring the above plasmid produce approx. 1 mg rec-hTNF per liter culture if grown in LB medium until the early stationary phase (Mai et al., manuscript in prep.).

2.4. *Purification and assay of tumor necrosis factor*

Recombinant TNF was purified by a combination of ammonium sulfate precipitation, controlled pore glass and hydrophobic interaction chromatographies and FPLC (Mai et al., manuscript in prep.). If necessary, the last traces of LPS were removed by affinity chromatography on polymyxin B-agarose. The final product yielded a single electrophoretic band of approx. 17 kDa, was at least 95% pure (as determined by silver staining of SDS-polyacrylamide gels), had a specific activity of at least 20 million U/mg and contained less than 0.125 U of endotoxin per mg protein (measured by the LAL-Pyrogent assay, Wittaker Bioproducts, Walkersville, MD).

L929 cells were grown in DMEM (Serva, Heidelberg) with 5% serum. Supernatants assayed for the presence of TNF were extracted with chloroform to remove RU 38486 before dialysis and concentration.

Bioassay of TNF was based on its cytotoxicity, measured on mouse L929 tumor cells, in the presence of 1 μg/ml actinomycin-D, at exactly 37°C [1,23]. The TNF sensitivity of normal mouse 3T3 cells was assayed in the presence of 0.125 and 0.25 μg/ml actinomycin-D. These cells tolerated up to 10,000 U TNF at 37°C, in the absence of mifepristone.

Killing of cells was assessed via the uptake of Neutral red vital dye. The amount of TNF required to mediate the half-maximal cytotoxicity of L929 cells was assigned a value of 1 U [1,23].

The tissue (liver, spleen) was minced and sonicated, and membranes were pelleted by centrifugation and resuspended by brief sonication. TNF was titered in both the supernatant and membrane fractions.

2.5. *TNF producing animal cells*

P388 mouse myeloid tumor cells and TNF-producing M9 human epithelial tumor cells (derivatives of HeLa cells) were grown in DMEM with 5% serum. P388 cells do not produce TNF unless activated by the presence of endotoxin or exogenously added TNF. M9 cells harbor a DNA construct containing the human TNF-α gene under the control of the SV40 enhancer, and therefore express TNF constitutively, at high level.

2.6. *Data analysis*

The comparisons of the means after analysis of variance were carried out by the method of Scheffe. Statistical significance was accepted at $P < 0.05$. Survival data were analysed for statistical significance by the Fisher exact test.

3. RESULTS

RU 38486 greatly increased the toxicity of hTNF both in tissue cultures and in animals. The highly sensitive mouse L929 tumor cells were killed by a 2–3 times lower TNF concentration, if RU 38486 was also present. 3T3 normal mouse fibroblast, which is highly resistant to TNF, became sensitive in the presence of the drug (Fig. 1). Intravenous injection of 2 μg/10 g body weight of hTNF, concurrently with 1 μg/g body weight of LPS killed 4 of 20 animals (80% survival), but when it was supplemented with RU 38486 treatment (1 mg) all of the 20 animals died (Table I). Injection of methylprednisolone (2 mg) concurrently with hTNF + LPS + RU resulted in significant protection (70% survival). It was earlier shown that bacterial endotoxin potentiates the toxicity of TNF [24], so that the simultaneous presence of otherwise innocuous amounts of LPS and

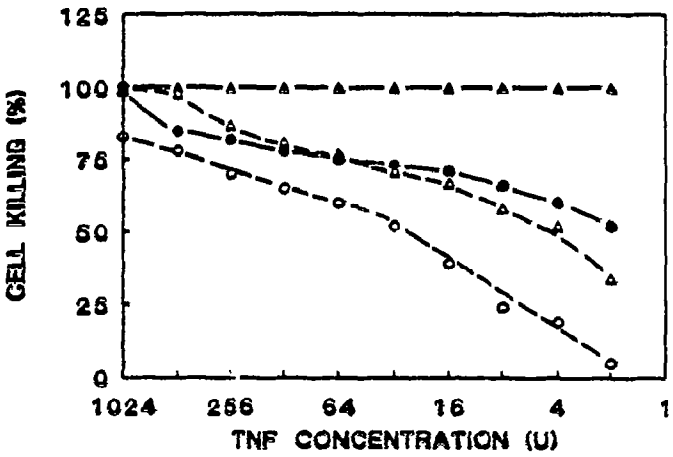

Fig. 1. The effect of RU 38486 on the TNF-sensitivity of 3T3 cells. Mouse 3T3 normal fibroblasts were grown on microtiter plates. The cultures were treated with liposomes or liposome entrapped mifepristone (40 μg/ml) before the exposure to different concentrations of TNF. 0.125 or 0.25 μg/ml actinomycin-D was added to parallel cultures. (▲) 0.25 μg/ml actinomycin-D + RU; (●) 0.125 μg/ml actinomycin-D + RU; (△) 0.25 μg/ml actinomycin-D; (○) 0.125 μg/ml actinomycin-D.

TNF triggers lethal shock. In our experiments, TFN + RU gave 70% survival, but if combined with LPS treatment, the survival dropped to 0%.

The effect of RU 38486 was not limited to the increased TNF sensitivity of cultured cells and animals. The effect of the drug on TNF production was also demonstrated in vitro. In M9 cells, which produce hTNF (as a result of genetic transformation with the hTNF gene), the TNF synthesis rate was more than doubled as a result of RU 38486 treatment (Fig. 2). In mouse P388 myeloid tumor cells the regulation of the

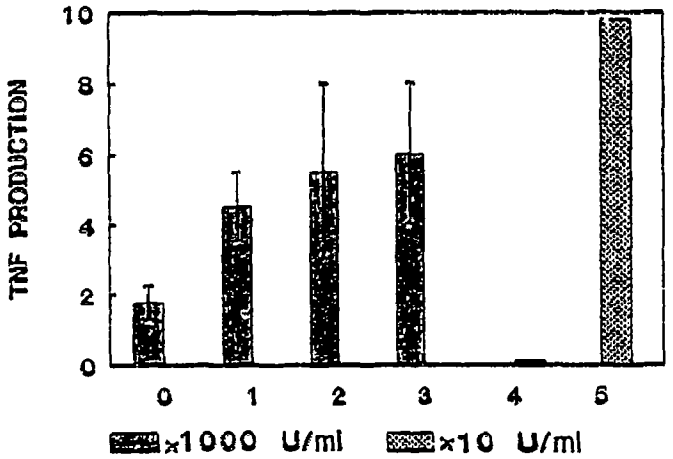

Fig. 2. The effect of RU 38486 on the TNF production of M9 and P388 cells. The cells were grown in DMEM. TNF was collected from the medium and titered as described in Methods. The figure shows daily production of confluent cultures, 10^6 cell/ml. 0 = M9 cells, grown without mifepristone; 1, 2 and 3 = M9 cells, grown in the presence of 6, 20 and 60 μg/ml mifepristone, respectively. P388 cells were grown in the absence (4) and the presence (5) of 40 μg/ml mifepristone.

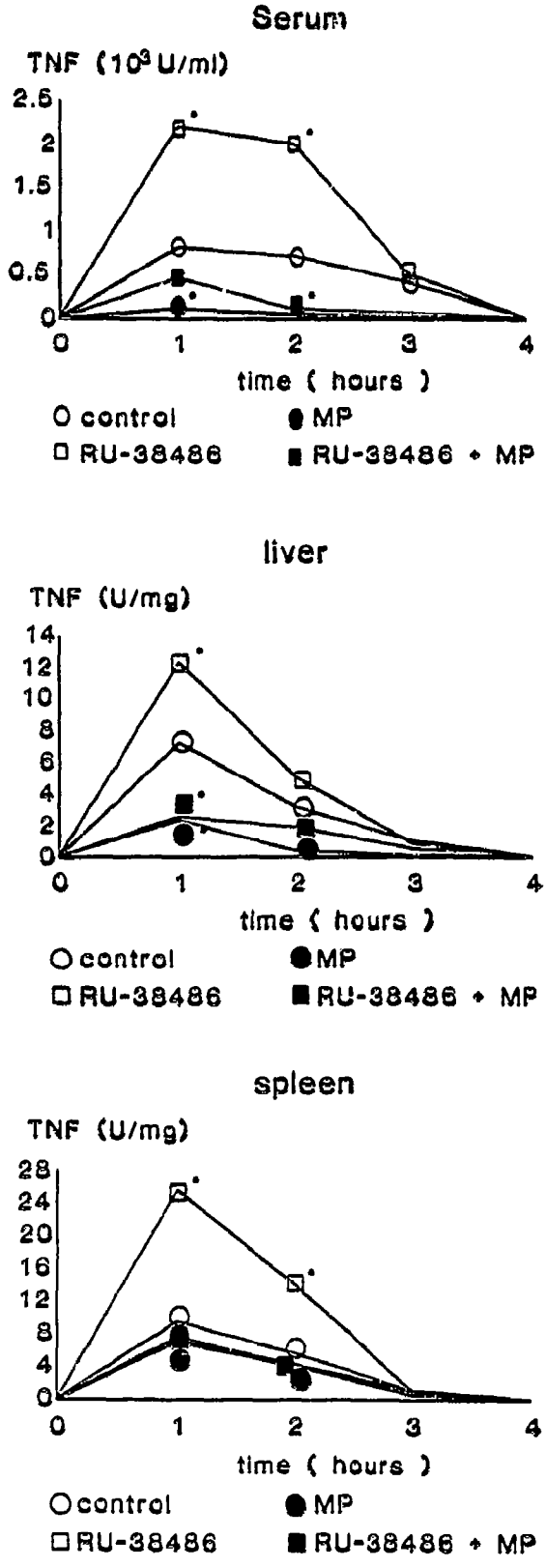

Fig. 3. The effect of RU 38486 on the endotoxin-induced TNF production in the blood (A), liver (B) and spleen (C). Animals were given liposomes alone, RU 38486 (1 mg), methylprednisolone (MP) (2 mg) or RU 38486 (RU) + MP intravenously just before the endotoxin challenge (1 μg/g). TNF activity was measured 1, 2, 3 and 4 h after LPS treatment. Means of results on 10 mice per group.

TNF-α gene was unaltered. These cells did not produce TNF without induction by LPS, TNF or phorbol esters. The presence of RU 38486 alone induced the synthesis of relatively high amounts of TNF in these cultures. The effect of RU 38486 on the rate of LPS-induced TNF production in the serum, liver and spleen is shown in Fig. 3A,B,C. At the time of peak response (one hour after LPS injection) RU 38486 had significantly increased the concentration of TNF in the serum, liver and spleen as compared to the control or methylprednisolone-treated groups. Methylprednisolone significantly decreased LPS-induced TNF production and completely abolished the effect of RU 38486. Neither RU 38486 nor methylprednisolone induced TNF production (data not shown).

4. DISCUSSION

TNF-α (cachectin) is a macrophage-derived peptide hormone released in response to different stimuli, including bacterial LPS. It has been implicated as a principal mediator in septic and endotoxin shock. Several lines of evidence have so far indicated that the pituitary-adrenal axis has an important part in regulating the TNF activity. Adrenalectomy sensitizes mice to the lethal effect of TNF, and that sensitization is abolished after addition of dexamethasone [25]. Furthermore, in adrenalectomized or hypophysectomized mice the LPS-induced serum TNF concentration remains at a high level as compared to normal animals [21]. In in vitro models, glucocorticoids inhibit the synthesis of TNF and another important septic shock mediator, IL-1, at the levels of both transcription and translation [19,26,27]. Moreover, in experimental animals dexamethasone protects against lethality induced by TNF [28]. Our results prove that RU 38486 significantly increases the endotoxin-induced TNF levels in the serum, liver and spleen and the drug can induce TNF synthesis in myeloid cells, even in the absence of endotoxin. These observations explain the shock-sensitizing effect of this compound, and suggest the involvement of endogenous

Table 1

Influence of RU 38486 on the lethal effect of TNF-α

Treatment	live/total	survival rate (%)	statistics
(1) TNF	20/20	100	
(2) LPS	20/20	100	
(3) TNF+LPS	16/20	80	3 vs. 1 NS
(4) TNF+RU	14/20	70	4 vs. 1 $P < 0.05$
(5) LPS+RU	20/20	100	5 vs. 2 NS
(6) TNF+LPS+RU	0/20	0	6 vs. 3 $P < 0.001$
(7) TNF+LPS+RU+MP	14/20	70	7 vs. 6 $P < 0.001$

Animals were given LPS (1 μg/g), TNF (2 μg/10 g), RU 38486 (1 mg) and MP (2 mg) intravenously. Numbers of survivors were recorded after 48 h. NS = not significant.

glucocorticoids in the regulation of TNF expression. RU 38486 acts on the receptor level, and thus the increased TNF production seems to be mediated via the glucocorticoid receptors. This is corroborated by the observation that methylprednisolone completely abolished the effect of RU 38486 on the production of TNF.

Our experiments revealed that RU 38486 enhances the toxic effects of hTNF in both cell cultures and experimental animals. It increases the expression of the TNF gene in cells producing TNF and simultaneously decreases the tolerance of cells to TNF by interfering with the protective effect of endogenous glucocorticoids. A recent receptor [29] demonstrated that TNF causes abortion in pregnant mice and destroys embryonic cells in combination with IFN-τ. It is conceivable that, in addition to its action as an antiprogesterone, the increased susceptibility of the fetal tissues to endogenous TNF may also play a role in the abortogenic effect of mifepristone.

Our results concerning the effect of the new glucocorticoid antagonist on systemic TNF production and toxicity confirmed the important role endogenous glucocorticoids play in the control of the immunopathological processes caused by high levels of certain lymphokines.

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