ONCOLOGY

Effect of the Dose of Dexamethasone and Cell Population Composition on the *in Vitro* Production of Tumor Necrosis Factor and Interleukin-6 by Human Peripheral Blood Mononuclears

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 121, No. 6, pp. 667-672, June, 1996
Original article submitted March 27, 1995

In vitro experiments demonstrate that various doses of dexamethasone not only inhibit production of interleukin-6 and tumor necrosis factor but also markedly stimulate it in cells of the total pool of human peripheral blood mononuclears. Production of tumor necrosis factor in the presence of dexamethasone is stimulated in all donors, while production of interleukin-6 is strongly inhibited, usually in a dose-dependent manner.

Key Words: *glucocorticoids; interleukin-6, lipopolysaccharide; peripheral blood mononuclears; adherent cells; tumor necrosis factor*

The regulation of inflammatory processes by glucocorticoid hormones is interesting not only for clinicians, who consider them primarily as drugs, but also for immunologists and molecular biologists, who use them as a tool for modulating transcription of numerous genes involved in the immune response. Mononuclear phagocytes are the first cells which respond to the action of damaging factors by producing various cytokines, proteases, free radicals, and active arachidonic acid metabolites (prostaglandins, leukotrienes, etc.) released from the cell membrane due to the action of phospholipase A_2. This is accompanied by the surface expression of inducible receptors and molecules of adhesion [16].

Glucocorticoid hormones are able to modulate *in vitro* virtually all functions of mononuclear phagocytes: to lower the intensity of free radical oxidation, the activity of proteolytic enzymes [16], the production of cytokines such as tumor necrosis factor-α (TNF-α) [5], interleukin-1 (IL-1), interleukin-6 (IL-6) [14,19], and interferons [16], and to diminish the production and activity of phospholipase A_2 [15].

In the whole organism, the activation of mononuclear phagocytes and production of TNF, IL-1, and IL-6 lead to activation of the hypothalamus-pituitary-adrenal system [9]. The surge of glucocorticoid hormones in turn triggers a negative feedback inhibiting IL synthesis and suppressing other functions of mononuclear phagocytes. Some pathological states, for instance, septic (endotoxic) shock, are characterized by disturbances in the physiological regulation of the IL-corticosteroid chain, so that neither elevation of endogenous nor high doses of exogenous glucocorticoids are able to lower the high level of TNF and IL-1. The latter are known to be the main pathogenetic factors of this extremely severe syndrome.

Study of the regulation of IL production opens up prospects for pharmacological control of the ac-

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0007-4888/96/0006-0602$15.00

tivity of mononuclear phagocytes. The effect of glucocorticoids on the IL-producing cell may depend on the stage of activation and differentiation, the microenvironment, and the culture medium. For instance, it has been shown that in a serum-free medium, combined pulsed treatment of peripheral blood mononuclears (PBM) with a mitogen and hormones suppressing the IL-2 gene transcription in doses of 10^{-8}-10^{-10} M may stimulate expression of the IL-2 gene [1].

The object of the present study was to investigate the effect of a wide dose range of dexamethasone (DM) on the production of TNF and IL-6 by mononuclears and the effect of the composition of the cell suspension on this production.

MATERIALS AND METHODS

Mononuclear cells were isolated from peripheral blood of healthy donors by gradient centrifugation using a Ficoll-Verographin mixture. The cells were washed twice with phosphate-buffered isotonic NaCl and resuspended in serum-free RPMI-1640 medium (ICN) supplemented with 20 μg/ml gentamicin, 2 mM L-glutamine, and 2 mM HEPES. The TNF- and IL-6-containing supernatant was obtained by incubating the cells with 10 μg/ml lipopolysaccharide from meningococcae of the A serogroup (G. N. Gabrichevskii Moscow Research Institute of Epidemiology and Microbiology) alone or in combination with varying doses of DM (10^{-6}-10^{-12} M). The PBM suspension (10^6 cells/ml) was placed in a 24-well plate (Nunc) in a volume of 1.5 ml/well and incubated for 3.5 h at 37°C in a humidified atmosphere containing 5% CO_2 with lipopolysaccharide and DM. The cells were washed with serum-free medium by centrifuging the plate and then 1.5 ml culture medium were added to some wells, while in others the cells were carefully resuspended and the suspension of nonadherent cells was transferred to empty wells, while the wells with adherent cells were filled with medium. These three cultures differing in cell composition (nonseparated PBM suspension, nonadherent cells, and adherent cells) were incubated for 18 hours, after which the supernatant was collected and stored at -20°C.

The content of IL-6 in the supernatant was measured using IL-6-dependent D6C8 heterohybridoma, obtained by fusing X653 murine myeloma with splenocytes from August rats preimmunized with lipopolysaccharide [2]. The supernatant was preliminarily inactivated at 56°C for 30 min. Serial dilutions of the supernatant were incubated with D6C8 hybridoma cells (5×10^4 cells/well, 48 h, 37°C, 5% CO_2) in 96-well flat-bottom plates (Nunc) in 200 μl RPMI-1640 medium containing 5% dialyzed human serum of blood group IV (AB). ^{3}H-thymidine (40 kBq/well) was added 4 h prior to the end of incubation. After the incubation was terminated the cells were transferred to glass filters and the incorporation of the label was measured using a liquid scintillation counter. Titration curves of the samples and a standard preparation were analyzed using probit analysis [3]. Recombinant human IL-6 (code 89/548) supplied by the National Institute for Biological Standards and Control (Great Britain) served as the standard. The content of IL-6 in the supernatants was expressed in international units (IU).

TNF activity was evaluated by the degree of lysis of TNF-sensitive L-929 murine sarcoma cells [8]. The L-929 cells were cultured at 37°C in 96-well flat-bottom plates (3×10^4 cells/well) in medium 199 on Hanks solution (Institute of Polyomyelitis, Russian Academy of Medical Sciences, Moscow) in the presence of 10% inactivated cattle serum until a monolayer formed. Thereafter the medium was decanted and 100 μl of serial dilutions of the supernatants (1:2-1:8) and 100 μl of fresh medium with actinomycin D (2 μg/well, Sigma) were added to each well. The cells were incubated for 16-20 hours at 37°C and 5% CO_2. The reaction was assessed by crystal violet staining. To this end the cells were incubated for 15 min with 0.2% crystal violet solution (Sigma) in 2% ethanol, and then washed in running water and dried. The optical density of the stained cells was measured at 540 nm using a vertical-beam spectrometer (Multiscan). Human recombinant TNF-α (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow) was used as the standard. Titration curves were analyzed by probit analysis and the content of TNF was expressed in pg/ml.

TABLE 1. Cell Distribution in Studied Suspensions

Cell suspension	Type of cell, %		
	lymphocytes	monocytes	granulocytes
Total PBM	86.4	12.0	1.7
Adherent cells	62.3	25.5	12.2
Nonadherent cells	95.9	3.3	0.9

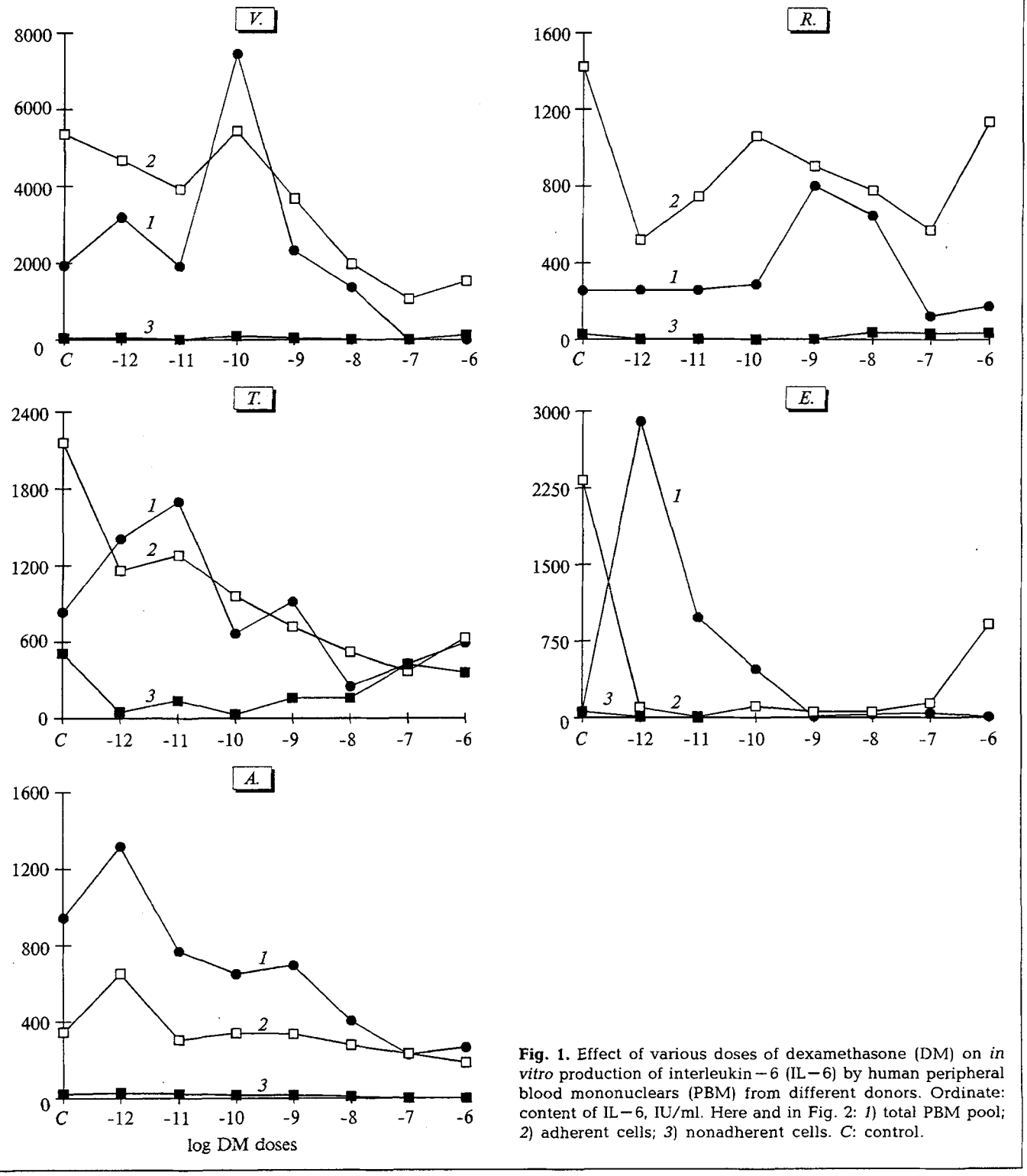

Fig. 1. Effect of various doses of dexamethasone (DM) on *in vitro* production of interleukin-6 (IL-6) by human peripheral blood mononuclears (PBM) from different donors. Ordinate: content of IL-6, IU/ml. Here and in Fig. 2: *1*) total PBM pool; *2*) adherent cells; *3*) nonadherent cells. *C*: control.

The composition of cell suspensions from the blood of donor *R.* was analyzed on an EPICS ELITE laser flow cytofluorometer (Coulter Electronics). To this end the cells were harvested with a cell scraper. The size of cells and granulation of the cytoplasm were evaluated by small-angle and perpendicular light scattering, respectively, and the subpopulation composition was analyzed by indirect immunofluorescence using monoclonal antibodies to CD3, CD14, and CD20 antigens (Coulter Clone). The data were processed statistically using Multigraph software (Coulter Electronics).

RESULTS

Simultaneous application of two modes of light-scattering (small-angle and perpendicular) to the analysis of peripheral blood cells is known to make it possible to identify 3 populations among human leukocytes: lymphocytes, monocytes, and granulocytes. When we used this approach to study cell distribution in the total PBM population and in the suspensions of adherent and nonadherent cells, we found the population of adherent cells to be greatly enriched with monocytes and B cells, while the majority of nonadherent cells were lymphocytes (Table 1). In the total PBM suspension T ($CD3^+$) and B ($CD20^+$) cells constituted 59.8 and 8.3% of lymphocytes, respectively. Among lymphocytes of the population of adherent cells T and B cells constituted 23.8 and 70%, respectively, while in the population of nonadherent cells the respective parameters were 70.4 and 16%. Eighty-nine percent of cells identified as monocytes in the total PBM population exhibited the $CD14^+$ phenotype, while in monocytes of the adherent population this phenotype was observed in 79.4% of cells.

After 18-hour culturing in the serum-free medium, the accumulation of IL-6 was shown to be more intensive in wells containing adherent cells (Fig. 1, except for donor *A.*). In wells containing the total pool of PBM the level of IL-6 was lower. The minimal production of IL-6 was noted in nonadherent cells. Thus, the level of IL-6 measured in the supernatant of the total PBM pool may be considered to be a result of the interaction between adherent and nonadherent cells, the nonadherent cells exerting an inhibitory effect (in donor *A.*, on the contrary, we noted a mutual stimulating interaction between adherent and nonadherent cells).

Under the action of a wide range of DM doses the dynamics of IL-6 accumulation was very differ-

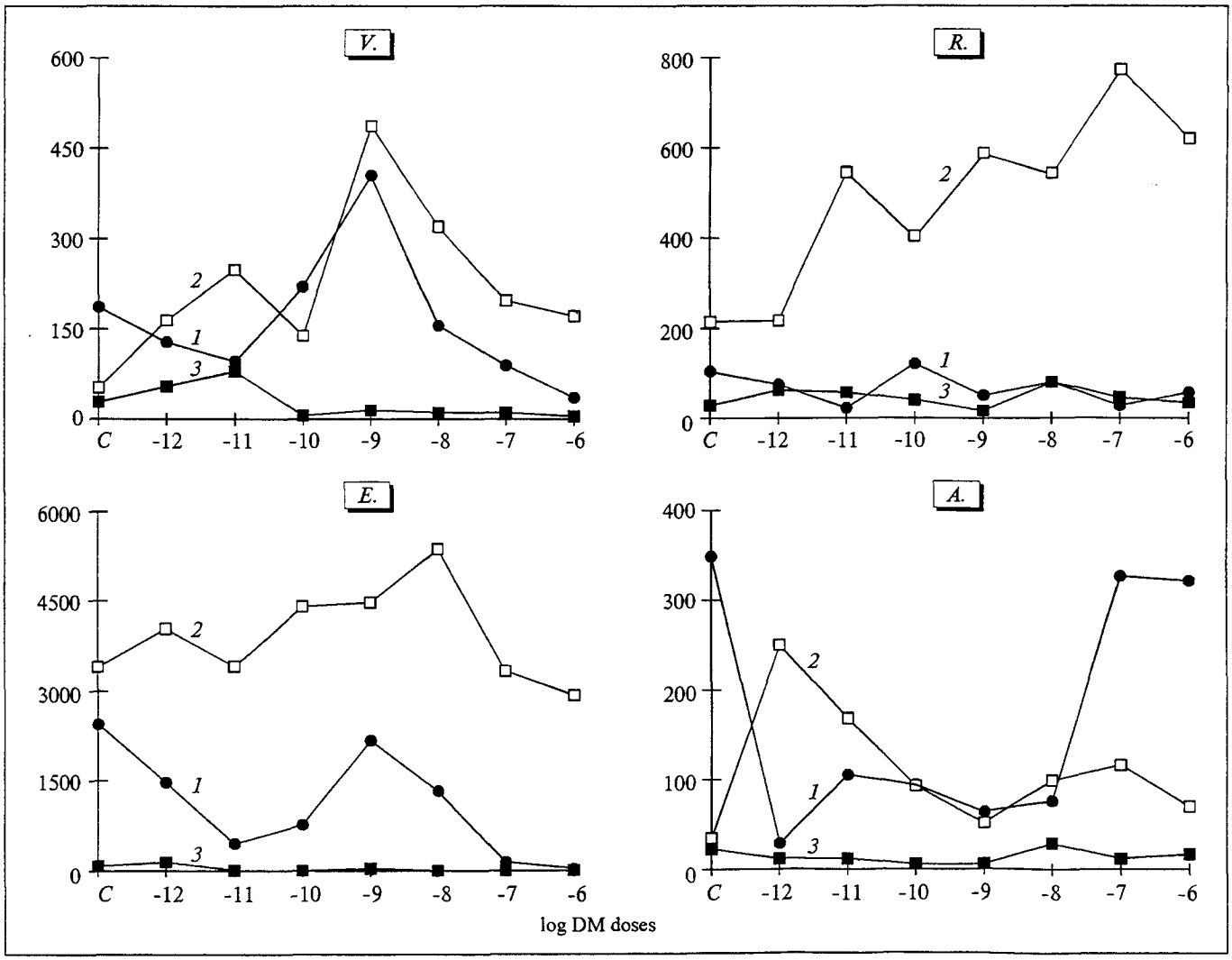

Fig. 2. Effect of various doses of dexamethasone (DM) on *in vitro* production of tumor necrosis factor (TNF) by human peripheral blood mononuclears (PBM) from different donors. Ordinate: content of TNF, pg/ml.

ent. In adherent cells it markedly dropped, generally in a dose-dependent manner. Only in one donor (*A.*) did we observe a stimulation of IL-6 production by the lowest dose of DM (10^{-12} M, Fig. 1). The meager production of IL-6 by nonadherent cells was even further lowered by DM. On the other hand, in the total PBM pool low and medium doses of DM (10^{-12}-10^{-9} M) markedly stimulated, while high doses (10^{-7}-10^{-6} M) suppressed IL-6 production in all 5 donors. It may be assumed that apart from inhibiting IL-6 production in adherent and nonadherent cells, DM in the total mononuclear suspension diminishes the effect of factors of cell-to-cell cooperation normally inhibiting IL-6 production.

The TNF assay in the same supernatants (only from 4 donors) showed that in practically all applied doses DM stimulated TNF production by adherent cells. Only in donor *E.* did high doses of DM (10^{-7}-10^{-6} M) inhibit the accumulation of TNF (Fig. 2). The minor production of TNF by nonadherent cells may be either slightly suppressed or slightly stimulated by various DM doses (Fig. 2). In donors *A.* and *E.* DM inhibited production of TNF by the total PBM population, while in the other two it was increased at low concentrations of DM: 10^{-10} M for donor *R.* and 10^{-9}-10^{-10} M for donor *V.* (Fig. 2).

It should be noted that the suppression of cytokine production has been reported by the majority of scientists studying the effect of glucocorticoid hormones. For instance, glucocorticoids have been shown to suppress lipopolysaccharide-dependent induction of IL-6 in human fetal Kupffer cells [11], fibroblasts, monocytes, and endothelial cells [19]. This suppression of transcription is effected through direct association of the p65 subunit of NFκB transcription factor (gene activation factor for IL-6 and other lymphokines) with activated glucocorticoid receptor [14]. Only Sironi and co-workers have demonstrated *in vivo* on mice that DM, like indomethacin, is able to stimulate high production of IL-6 even without lipopolysaccharide and does not suppress endotoxin-induced IL-6 production.

Ample data have been gathered on the regulation of TNF production. Some authorities have reported that glucocorticoids inhibit TNF production at the level of transcription and translation [5,10,16]. There are a number of other factors suppressing the synthesis of TNF by mononuclears, among them products synthesized by both monocyte-derived macrophages (prostaglandin E_2 [13], IL-6 [17], IL-10 [6,12], transforming growth factor-β2 [6]) and T helpers (IL-4 and IL-10 [6]). Moreover, TNF activity in the supernatant is blocked by soluble TNF receptors sloughed off the cell surface into the plasma or culture medium by proteases [7]. Synthesis of prostaglandin E_2 [13,15] and many IL suppressors of TNF production [10] is inhibited by glucocorticoids, the doses inhibiting production of such cytokines as IL-1, IL-4, IL-6, IL-10, and γ-interferon being much lower than those inhibiting production of TNF (as well as IL-2 and IL-3) [10]. It may be assumed that under these experimental conditions the inhibition of the synthesis of TNF suppressors surpasses the inhibition of synthesis of TNF itself. Such conditions may serve as a model for investigating the ineffective suppression of TNF synthesis by glucocorticoids at the level of transcription and translation (other transcription factors may interfere with the interaction between hormone-activated receptors and NFκB and AP-1 transcription factors [4]) or the more pronounced effect of TNF on target cells due to the absence of soluble TNF-blocking receptors in the supernatant.

The authors are grateful to V. I. Kuvakina for kindly supplying the lipopolysaccharide preparation.

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