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The influence of glucocorticoids with different activity duration on cytokine production by mononuclear cells and their immune dialogue with HT-29 cells

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Abstract
Background: The introduction of glucocorticoids (GCS) in medical practice made a revolution in the treatment of auto-immune diseases and inflammatory disorders. GCS repress inflammatory processes by their ability to modulate cytokine production by macrophages that are the main cohort of cells invading inflammatory sites. This effect is concentration- and activity-duration dependent and accordingly GCS are designated as brands with short, intermediate and long activity. The aim of the study was to examine the effect of hydrocortisone (HC), prednisone (Pred) and dexamethasone (Dexa) as representatives of the three groups respectively, on the capability of human peripheral blood mononuclear cells (PBMC) to produce cytokines and to detect their influence on the immune dialogue between PBMC and HT-29 cells from a human colon carcinoma line.
Methods: Not-stimulated PBMC or cells stimulated with either LPS or PMA/ionomycin were incubated without or with one of the tree GCS at two concentrations calculated according to their relative potency. The production of TNFα, IL-1β, IL-6, IFNγ, IL-2, and IL-10 was assessed. In another set of experiments PBMC were co-cultured with HT-29 human colon carcinoma cells in absence or presence of GCS and the secretion of the abovementioned cytokines was evaluated.
Results: Not-stimulated PBMC did react to Pred (intermediate acting) and Dexa (long-acting) by inhibited TNFα secretion at the lower concentrations only. Stimulated PBMC incubated separately with the three GCS showed repressed production of all cytokines except for IL-6 that was not affected by HC and IL-10 whose production was influenced by Pred only. PBMC co-incubated with HT-29 cells showed inhibited secretion of TNFα and IL-1β under the effect of all three GCS, whereas that of IL-6 and IFNγ was constrained by Pred and HC.
Conclusions: The findings indicate that GCS of the three groups suppress the production of inflammatory cytokines rather comparably and only prednisone exerts this effect on the generation of the pro-inflammatory IL-6 and the anti-inflammatory IL-10. These observations may help reaching a suitable decision as for the type of GCS to be used in certain medical conditions.

Keywords: glucocorticosteroids, cytokines, mononuclear cells, HT-29 cells, colorectal cancer

Introduction

The introduction of synthetic corticosteroids (GCS) for clinical use was ensued by a great improvement in the treatment of a large inventory of illnesses including acute and chronic stress conditions, inflammatory processes and immune related diseases [1]. Macrophages, being one of the pivotal components of the immune system, are readily affected by corticosteroids. The capacity of GCS to regulate cytokine production by monocytes and macrophages is of utmost importance in cases of inflammation. GCS not only prevent monocytes' activation by pathogens but also stir up their differentiation from pro- to anti-inflammatory monocytes capable to produce mediators limiting inflammation [2]. Studies have shown that on one hand GCS have the ability to inhibit the expression of a great number of pro-inflammatory cytokines, such as IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-12, TNFα and IFNγ, while on the other hand they stimulate the generation of the anti-inflammatory cytokines - IL-10 and IL-1ra [1, 3-6]. Notable, while the release of IL-10 by peripheral blood macrophages in asthmatic individuals remained unaffected by GCS, its production by alveolar macrophages was stimulated indicating that professional macrophages respond differently to the drugs [4]. 1μM of dexamethasone inhibited IL-10 and IL-17 production by lung lymphocytes of asthmatic patients by 50% or less, and that of IL-2, IL-13 and IFNγ was repressed even more, indicating that the production of cytokines by immune cells of asthmatic patients is not utterly controlled by GCS [7]. Murine macrophages treated with GCS showed inhibited production of IL-12, a cytokine with high potency for stimulation of IFNγ release and for constrain of IL-4 synthesis by T cells [8]. GCS regulate cytokine production by human mononuclear cells through a number of mechanisms detailed by Liu *et al* [9]. Considering the close relationship between inflammation and

cancer development the anti-inflammatory property of the GCS qualify them as significant contributors to the list of therapeutic anti-cancer regiments. In addition to their activities as analgesics in patients with severe bone or neuropathic pains they express beneficial effect in prevention of certain tumors' development [10]. Reviewing the subject Raybum *et al* [11] conveyed experiments in the field, demonstrating that administration of dexamethasone to mice exposed to tobacco smoke reduced the incidence for developing lung tumors by more than 60%, and enhanced the therapeutic results in animals with glioma, breast and colon cancer receiving standard chemotherapy. With institution of GCS in clinical practice the number of synthetic GCS brands rapidly increased. It has been established that the beneficial effect of GCS on various morbid conditions is concentration-dependent. It is conceivable that this mode of activity will be reflected on the immune properties of the human peripheral blood mononuclear cells (PBMC) and particularly on their capacity for cytokine production. In a previous study we have found that budesonide reduced concentration-dependently the proliferation of PBMC and the generation of TNFα, IL-1β, IL-2 and IL-6 [12]. In addition to concentration-dependence the potency of synthetic GCS differ by their duration of activity. Consequently, they have been designated as short-, intermediate- and long-acting, represented generally by hydrocortisone, prednisone and dexamethasone respectively [13]. Bearing in mind these GCS attributes the subject of the present study was to examine the capacity for cytokine production by human peripheral blood mononuclear cells (PBMC) under the effect of GCS from the three duration-activity categories. In addition, the possibility that GCS with different extent of activity may modulate the immune cross-talk between PBMC and HT-29 cells from a human colon cancer cell line was evaluated.

Materials and Methods

Glucocorticoids

Glucocorticoids (GCS) were selected according to their relative potency. Hydrocortisone (HC) was chosen as short-acting, prednisolone (Pred) as intermediate-acting and dexamethasone (Dexa) being the long-acting one. Hydrocortisone (Solu-Cortef sodium-succinate 100 mg powder (Pfizer PFE Pharmaceutical, NV/SA, Puurs, Belgium) was dissolved in 1 ml of distilled water. Pred (methylprednisolone, as sodium succinate, Act-O-Vial of Solu-Medrol Pfizer PFE Pharmaceutical, Belgium NV/SA, Puurs, Belgium) containing 125 mg /vial, was diluted with 2 ml of distilled water. Dexa (sodium phosphate, 10mg/ml solution, West-Ward Pharmaceutical Corp. USA) was diluted in 0.9% NaCl solution. Further dilutions for all steroids were carried out with 0.9% NaCl solution. Since HC physiological plasma level is 0.2μg/ml [14], the concentrations of the other glucocorticoids were calculated according to their equivalent potencies. HC was added at final concentrations of 0.2μg/ml (HC-L) and 2μg/ml (HC-H). Pred was added at 0.05μg/ml (Pred-L) and 0.5μg/ml (Pred-H). Dexa-at 0.0075μg/ml (Dexa-L) and 0.075μg/ml (Dexa-H). All drugs were added at the onset of cultures at a final volume of 10μl/ml.

Cell preparation

Peripheral blood mononuclear cells (PBMC) were isolated from adult donors' venous blood after signing an informed consent containing an agreement to use for research components of their blood not required for therapeutic purposes. The cells were separated by Lymphoprep-1077 (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation, washed twice in phosphate buffered saline (PBS) and suspended in RPMI-1640 medium (Biological Industries, Beith Haemek, Israel) containing 10% heat inactivated fetal bovine serum (FBS) and designated as complete medium (CM).

Colon cancer cells

HT-29 human colon cancer cells were obtained from American Type Cultural Collection, Rockville, MD. The cells were maintained in CM containing Mc-COY'S 5A medium (Biological Industries Co, Beth-Haemek, Israel), supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and antibiotics (penicillin, streptomycin and nystatin-Biological Industries Co, Beth-Haemek, Israel). The cells were grown in T-75 culture flasks at 37 ^{o}C in a humidified atmosphere containing 5% CO2.

Effect of glucocorticoids on cell proliferation

The effect of GCS on PBMC and HT-29 cell proliferation was examined applying XTT proliferation assay kit (Biological Industries, Beith Haemek, Israel). In short: 0.1 ml aliquots of PBMC or HT-29 cells obtained after trypsinization and suspended at 10^5/ml in appropriate CM were added to each one of 96 well plates and incubated for 24 hrs in the absence or presence of GCS added at the onset of cultures at concentrations as indicated. At the end of the incubation period the cells were stained according to the manufacturer's instructions. The plates were incubated for additional 3 hrs at 37^{o}C in a humidified incubator containing 5% CO_2 and the absorbance was measured at 450 nm using an ELISA reader.

Effect of glucocorticoids on cytokine production

0.5 ml of PBMC ($4x10^6$/ml of CM) was incubated with an equal volume of CM without or with 40 ng/ml lipopolysaccharide (LPS, E. coli, Sigma) or with 1 μg/ml of phorbol meristate acetate (PMA) and 0.5 μg/ml of ionomycin (Sigma, Israel) or with 0.5 ml of HT-29 colon cancer cells ($4x10^5$/ml of CM) suspended in appropriate CM. All GCS were added at the onset of cultures in a volume of 10 μl/ml. The cultures were incubated for 24 hrs at 37^{o}C in a humidified atmosphere containing 5% CO2. At the end of the incubation period the cells were removed by centrifugation at 250g for 10 min., the supernatants were collected and kept at -70^{o}C until assayed for cytokines content.

Cytokine content in the supernatants

The concentration of TNFα, IL-1β, IL-2, IL-6, IFNγ, and IL-10 in the supernatants was tested using ELISA kits specific for these cytokines (Biosource International, Camarillo, CA) as detailed in the guide-line supplied by the manufacturer. The detection levels of these kits were: 15 pg/ml for IL-6 and 30 pg/ml for the remaining cytokines.

Statistics

A linear mixed model with repeated measures and the

assumption of compound symmetry was used to assess the effects of GCS on cytokine production by PBMC. SAS/Base and SAS/Stat vs 9.4 for Windows PC were used for this analysis. Paired t-test was applied to compare the level of cytokine produced with the different GCS at low and high concentrations and that found in control cultures (incubated without GCS). Probability values of $p<0.05$ were considered as significant. The results are expressed as mean ± SEM of 4 experiments.

Results

Effect of glucocorticoids on cell proliferation

24 hrs incubation of PBMC or HT-29 colon cancer cells with the two concentrations of the three GCS did not affect the proliferation rate as examined by XTT test (p>0.1).

Effect of glucocorticoids on cytokine secretion

HT-29 cells

No detectable amount of any of the cytokines examined could be found in supernatants collected from HT-29 cells incubated for 24 hrs at $2x10^5$/ml without or with the corticosteroids added at concentrations as indicated.

Non-stimulated PBMC

Incubation of non-stimulated PBMC with Pred or Dexa caused reduced secretion of TNFα (p=0.007 or p=0.029 respectively), whereas the production of the other cytokines was not affected. TNFα synthesis was 32% lower when PBMC were incubated with 0.05µg/ml or 0.5µg/ml of Pred (p<0.01) and by 26% when Dexa was applied at 0.0075µg/ml or 0.075 µg/ml ($p<0.05$), but was not affected by incubation with HC (p=0.067).

TNFα production (Fig.1).

While HC caused reduced production of TNFα by LPS-stimulated PBMC (p=0.0085), incubation with either Pred or Dexa had no effect on TNFα secretion (p=0.2, p=0.6 respectively). Incubation with HC at 0.2µg/ml or 2µg/ml showed 23% and 29% lower secretion of TNFα, respectively (p=0.025 and p=0.0089, respectively). The production of TNFα induced by HT-29 cells was inhibited following incubation with either HC (p=0.045), Pred (p=0.0006), or Dexa (p=0.02). At 2µg/ml of HC the synthesis of TNFα induced by HT-29 cells was inhibited by 23% (p=0.059), whereas following incubation with Pred at 0.05 and 0.5 µg/ml, HT-29-induced TNFα secretion was restrained by 25% (p=0.032) and 58% (p<0.001), respectively. The difference between the inhibitory effect of the two Pred concentrations added was statistically significant (p=0.008). Incubation with 0.0075 µg/ml and 0.075 µg/ml of Dexa caused reduced production of HT-29 -induced TNFα secretion by 22% and 37% ($p<0.05$).

IL-1β production (Fig.1)

A concentration dependent inhibition of LPS-induced IL-1β production by PBMC was found when PBMC were incubated with HC (p=0.0087), Pred (p<0.001) or Dexa (p=0.006). HC added at 0.2 µg/ml and 2 µg/ml caused reduced synthesis of IL-1β by 21% ($p<0.05$) and 30% ($p<0.005$), respectively, Pred at 0.05 µg/ml and 0.5 µg/ml lowered the secretion by 33% and 42% (p<0.01), respectively, and Dexa at 0.0075 and 0.075µg/ml by 19 % (p=0.09) and 37% (p<0.01), respectively. The secretion of IL-1β induced by HT-29 colon cancer cells was concentration dependent reduced when HC, Pred, or Dexa were applied (p=0.01, p<0.001 and p=0.04, respectively). Following incubation with the lower concentrations of the three glucocorticoids, the restrained secretion of IL-1β was not statistical significant. However, HC at 2µg/ml, Pred at 0.5µg/ml and Dexa at 0.075µg/ml caused reduced production of IL-1β by 30% (p=0.009), 31% (p=0.001) and 31% respectively (p<0.01). The difference between the inhibitory effect of the two Pred concentrations was statistically significant (p=0.008).

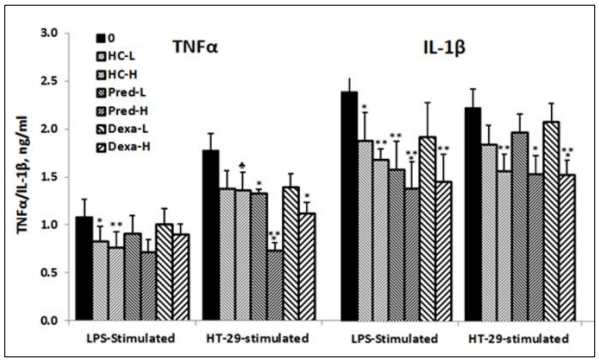

Fig 1: Effect of GCS on TNFα and IL-1β production

PBMC simulated with LPS (40ng/ml) or HT-29 colon cancer cells were incubated for 24 hrs without (0) or with the following GCS: hydrocortisone (HC), prednisone (Pred) or dexamethasone (Dexa) at low (L) or high (H) concentrations as indicated. Following the incubation period, supernatants were collected and the production of TNFα and IL-1β were examined using ELISA kits. Each column represents the mean results of four different samples. Bars represent SEM. Asterisks represent statistically significant difference from PBMC incubated without GCS (0) (*$p<0.05$; **p<0.01; *⁎*p<0.001; ♣p=0.06).

IL-6 secretion (Fig.2)

The secretion of IL-6 by PBMC stimulated by LPS or HT-29 colon cancer cells was not affected upon incubation with either HC (p=0.17, p=0.31 respectively) or Dexa (p=0.32, p=0.4, respectively). However, following incubation with Pred, IL-6 production by PBMC incited by LPS or HT-29 cells was reduced (p=0.0021, p=0.06, respectively). IL-6 production by LPS-induced PBMC was inhibited by 15% following incubation with both concentration of Pred ($p<0.005$) and by 20 % ($p<0.05$) when added to HT-29-induced IL-6 secretion.

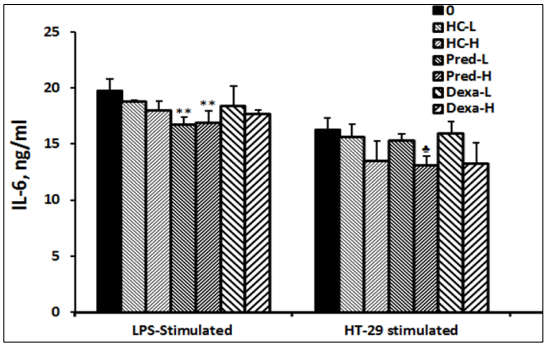

Fig 2: Effect of GCS on IL-6 production

PBMC simulated with LPS (40ng/ml) or HT-29 colon cancer cells were incubated for 24 hrs without (0) or with the following GCS hydrocortisone (HC), prednisone (Pred) or dexamethasone (Dexa) at low (L) or high (H) concentrations as indicated. Following the incubation period, supernatants were collected and the production of IL-6 was evaluated using ELISA kits. Each column represents the mean results of four different samples. Bars represent SEM. Asterisks represent statistically significant difference from PBMC incubated without GCS (0) (**p<0.01; ♣p=0.06).

IL-10 secretion (Fig.3)

The production of IL-10 induced by LPS was not affected following incubation with HC (p=0.28). Dexa at 0.075 µg/ml caused 36% reduction of its secretion (p=0.03). Pred at concentrations of 0.05µg/ml and 0.5 µg/ml inhibited the secretion of IL-10 by 34% and 37%, (p<0.002), respectively. The production of IL-10 induced by HT-29 colon cancer cells was not affected upon incubation with any of the GCS tested (p>0.1).

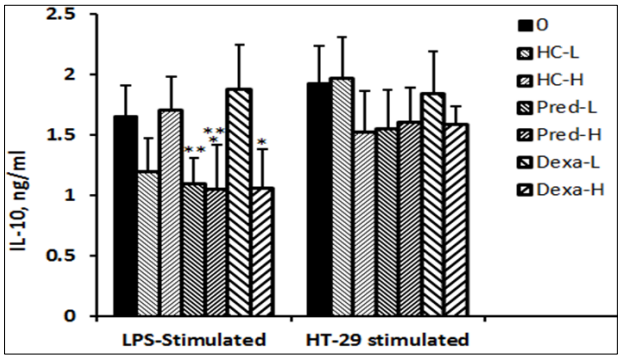

Fig 3: Effect of GCS on IL-10 production

PBMC simulated with LPS (40ng/ml) or HT-29 colon cancer cells were incubated for 24 hrs without (0) or with the followings GCS: hydrocortisone (HC), prednisone (Pred) or dexamethasone (Dexa) at low (L) or high (H) concentrations as indicated. Following the incubation period, supernatants were collected and the production of IL-10 was tested using ELISA kits. Each column represents the mean results of four different samples. Bars represent SEM. Asterisks represent statistically significant difference from PBMC incubated without GCS (0) (*p<0.05; **p<0.01; *$_*$*p<0.001).

IFNγ secretion (Fig.4)

The three GCS caused concentration dependent inhibition of IFNγ secretion by PBMC stimulated with PMA and ionomycin (p<0.005). At 0.2 and 2.0 µg/ml HC reduced IFNγ production by 17.5% and 20% (p<0.001), Pred at 0.05 and 0.5 µg/ml by 12% and 24% (p=0.03 and p=0.0017, respectively) and Dexa by 10% and 13% at 0.0075 and 0.075 µg/ml (p<0.001). The release of IFNγ by HT-29 stimulated PBMC was reduced only when HC was added (p=0.02) and was not affected by the two other GCS (p>0.1). HC caused 19% reduced secretion of this cytokine when added at both concentrations (p<0.05).

IL-2 production (Fig.4)

Upon incubation with low concentrations of HC, Pred or Dexa, the production of IL-2 induced by PBMC stimulated with PMA and ionomycin was reduced by 23%, 14% and 20%, respectively. When higher concentrations of the three drugs were applied the secretion of IL-2 was inhibited by 39%, 40% and 16.5%, respectively, however values did not reach statistically significance. The production of IL-2 induced by HT-29 colon cancer cells was not affected by 24 hrs of incubation with any of the drugs tested in the current study (p>0.1).

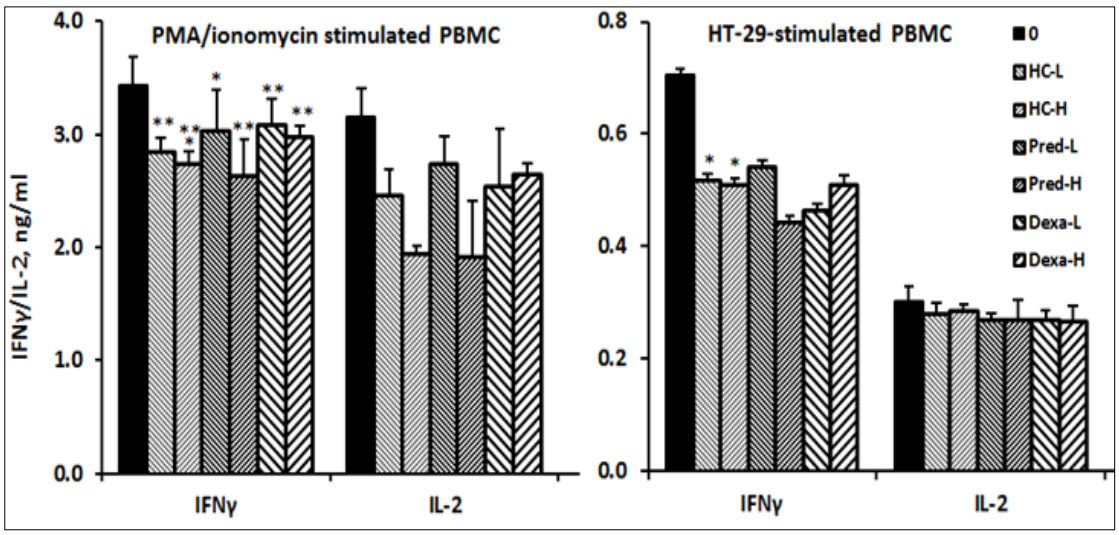

Fig 4: Effect of GCS on IL-2 and IFNγ production

PBMC simulated with PMA/ionomycin or with HT-29 colon cancer cells were incubated for 24 hrs without (0) or with the following GCS: hydrocortisone (HC), prednisone (Pred) or dexamethasone (Dexa) at low (L) or high (H) concentrations as Indicated. Following the incubation period, supernatants were collected and the production of IL-2 and IFNγ were detected using ELISA kits. Each column represents the mean results of four different samples. Bars represent SEM. Asterisks represent statistically significant difference from PBMC incubated without GCS (0) (*p<0.05; **p<0.01)

Discussion

The aim of the present study was to evaluate the ability of three GCS, each one with different length of activity, to modulate the capacity for cytokine production by human PBMC. From a clinical point of view the potency of steroids to affect inflammation is related to their activity-time – short acting drugs being the least potent [13]. Therefore we had to calibrate the GCS concentrations used in the study according to both their duration of activity and their relative potency. For this purpose we applied GCS concentrations based on the data published by Zoorov and Cender [13] who calculated the equivalent doses of the three GCS brands hereby used. We decided to apply two GCS concentrations – 0.2 µg/ml being in the range of the normal plasma cortisol values (0.1µg/ml -1.0 µg/ml) reported by Opert *et al* [14]. and 2.0 µg/ml, a level five times higher than the one observed in patients being at high-stress conditions. One way by which GCS restrain inflammation is promoting monocyte differentiation from M1 phenotype specialized for production of pro-inflammatory cytokines to alternatively activated M2 type cells, capable to produce anti-inflammatory cytokines [15, 16] and by increasing their phagocytic activity [17]. As the data in the present study demonstrate, the ability of PBMC to produce cytokines related to GCS concentration was evident for LPS stimulated cells and not for all cytokines examined. The dependence of LPS stimulated macrophage functions on GCS concentrations have been reported by Lim *et al* [5]. The authors have shown that low levels of GCS intensify their pro-inflammatory cytokine expression, whereas high concentrations inhibited that and additional macrophage activities such as NO production and phagocytic ability. The difference between Lim *et al* [5] results and our findings may be due to the fact that they worked with mouse peritoneal macrophages stimulated with LPS, whereas we used human not-stimulated PBMC. On the other hand, when we added GCS to LPS incited PBMC the immune cells reacted by markedly expressed inhibition of all cytokines except IL-10 whose production was suppressed by Pred only. In a study carried out with co-cultures of PBMC with synovial cells of patients with rheumatoid arthritis, addition of GCS inhibited the production of IL-17, IL-6, IL-1β, IFNγ and IL-10. Using different GCS concentrations the authors observed that the effect of GCS on cytokine production was expressed already at lower concentrations and it did not differ markedly from the higher ones [18]. In a previous work we have shown that budesonide, a semi-synthetic long-acting glucocorticoid inhibited **t**he secretion of TNFα, IL-1β, IL-1ra, IL-2, IL-10 and IL-6 by PBMC without relation to their activation [12]. The inhibitory effect of GCS on IL-10 production, an anti-inflammatory cytokine, may be one explanation for the tendency to infections in patients under GCS treatment. PBMC cultured in the presence of GCS exert initially suppressed secretion of IL-1β and TNFα and subsequently the production of a large number of pro-inflammatory cytokines. Remarkably, the release of the anti-inflammatory cytokine IL-10 was inhibited likewise [19].

It should be emphasized that GCS exert an ambiguous activity– although they are generally accepted as anti-inflammatory agents they may exert a pro-inflammatory effect. While their anti-inflammatory function is due to their ability to modulate cytokine production by suppressing cytokine encoding genes, it has been shown that they may increase the production of pro-inflammatory cytokines mainly IL-1β, as it has been shown in cases of acute stress [20]. In addition, in cases of injury GCS do not promote macrophage migration towards the affected site although they repress their differentiation to M1 pro-inflammatory phenotype [15].

The close relation between chronic inflammation and carcinogenesis on the one hand and the anti-inflammatory attributes of GCS on the other hand, prompted clinicians to include GCS in the therapeutic procedures in certain malignancies [21]. The activity of GCS in these cases is mediated through GCS receptors within the tumor microenvironment [22]. Notably, the anti-inflammatory activity of GCS proceeding via constriction of inflammatory cytokine production does not dependent on reduced cytokine receptor expression, which is even up-regulated when cytokine production is inhibited [19]. In addition, GCS induce alterations in mouse monocytes converting them to tumor associated macrophages linked with tumor immunosuppression [17, 23]. However, since GCS are involved in a relatively large number of metabolic functions and in modulation of the immune and other systems, caution has been forwarded that they may promote development and progression of cancer [24]. The results hereby detailed indicate that the three GCS separately added to PBMC co-incubated with HT-29 cells were able to inhibit the pro-inflammatory TNFα and IL-1β cytokine production being more expressed when the higher concentration was applied. In that setup HC and Pred inhibited IFNγ and IL-6 secretion respectively.

In conclusion, the fundamental observations from the present study are that all three GCS incubated with LPS stimulated PBMC exerted an inhibitory effect on almost all cytokines examined, except for IL-10 whose secretion was affected by Pred only. Moreover, GCS with different potencies acted differently. PBMC incubated with the short-acting HC inhibited the secretion of all cytokines except IL-6 and IL-10. The intermediate-acting Pred constrained all cytokine production excluding TNFα, whereas the long-acting Dexa affected IL-1β, IL-2 and IFNγ. Incubation of LPS stimulated PBMC in the presence of the intermediate acting Pred resulted in inhibited secretion of each one of the cytokines except for TNFα, while PBMC stimulated by HT-29 cells at the same culture conditions showed inhibited TNFα, IL-1β and IL-6 production. The long-acting Dexa behaved similarly to Pred when LPS triggered PBMC were examined, excluding the effect on IL-10. Its inhibitory effect was expressed only on the secretion of TNFα and IL-1β. It is suggested that when GCS are intended to be administrated for an achievement of a modulated cytokine production in cases of inflammation and control of cancer progression, the role of their concentration along with their activity-duration should be considered.

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