Suppression of human monocyte tumour necrosis factor-α release by glucocorticoid therapy: relationship to systemic monocytopaenia and cortisol suppression

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Aims Glucocorticoids suppress the release of tumour necrosis factor-α (TNF-α) by macrophages *in vitro* and cause monocytopaenia *in vivo*. These actions may contribute to anti-inflammatory and immunosuppressant effects. We therefore examined relationships between prednisolone concentration, suppression of monocyte TNF-α release, monocytopaenia and suppression of total cortisol concentration in healthy volunteers treated with a single dose ($1.5\ mg\ kg^{-1}$) of the glucocorticoid, prednisolone.

Methods Monocyte numbers, total cortisol concentration and prednisolone concentration were measured in blood samples collected over 48 h after the dose. Plasma from these samples was also tested for its capacity to suppress lipopolysaccharide-induced TNF-α release from monocytes in autologous whole blood cultures.

Results At 4 h after the dose, monocyte numbers in peripheral blood had fallen to a mean of 18% of the pre-dose level whilst plasma total cortisol had fallen to 9% of the pre-dose concentration. Monocyte numbers recovered in concordance with elimination of prednisolone and there was a significant relative monocytosis at 24 h. The recovery of plasma cortisol was delayed in comparison, with cortisol remaining significantly suppressed at 24 h. Plasma samples taken at 2 h after the dose (corresponding to peak plasma prednisolone concentration) suppressed the lipopolysaccharide-stimulated production of TNF-α by autologous blood monocytes to 27% of pre-dose control. Plasma collected at intervals over the 48 h from dosing also suppressed monocyte TNF-α release in relation to the prednisolone concentration therein. Suppression was largely reversed by the glucocorticoid antagonist, mifepristone. A similar relationship between prednisolone concentration and TNF-α suppression was observed when prednisolone was added to blood samples collected from the volunteers when they were drug-free.

Conclusions Blood concentrations of prednisolone achieved after a dose of $1.5\ mg\ kg^{-1}$ are sufficient to suppress monocyte TNF-α release and cause a biphasic change in peripheral blood monocyte numbers. Suppression of TNF-α is principally a direct glucocorticoid effect, rather than a consequence of other prednisolone-induced changes to blood composition.

Keywords: TNF-α, prednisolone, human, monocytopaenia, cortisol, pharmacokinetics, pharmacodynamics

Introduction

The monokine tumour necrosis factor-α (TNF-α) contributes to the pathogenesis of endotoxic shock, rheumatoid arthritis, multiple sclerosis and other inflammatory diseases [1–3]. Glucocorticoids are effective anti-inflammatory and immunosuppressive agents, with actions on most cells involved in inflammatory or immune responses [4]. *In vitro*, glucocorticoids suppress release of TNF-α and a wide range of other monocyte/macrophage activities [5–8]. Glucocorticoids also provide relative protection from lethality in experimental endotoxaemia, suggesting capacity to suppress TNF-α production *in vivo*, at least in the doses used experimentally [9, 10]. If similar actions occur during human therapy, they may contribute to the anti-inflammatory efficacy of glucocorticoids. Indeed, the concentrations measurable in blood during therapy with the glucocorticoid, prednisolone, can reach the level which would be expected to suppress monokine production *in vitro* [11]. However, whereas the free concentration of prednisolone would be expected to approximate the total concentration under experimental conditions, the free fraction of prednisolone in blood is concentration-dependent and may be only 5% of the total [11, 12]. This may be insufficient for a clinically-relevant effect on TNF-α release. Furthermore, prednisolone concentrations decline rapidly after a conventional oral dose, so effective concentrations may be present only transiently

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[11, 13]. There is no information on the duration of monocyte/macrophage response after transient exposure to glucocorticoids and, therefore, no means to predict whether any effects of glucocorticoids *in vivo* would persist as the drug was eliminated. Together, these factors may partially explain the failure of glucocorticoids to influence the outcome of other conditions in which TNF-α has a role in pathogenesis, including human endotoxic shock [14] and the failure of glucocorticoids to reproduce *in vivo* some monocyte effects which are demonstrable *in vitro* [15].

Nonetheless, glucocorticoids are effective in suppressing inflammatory activity in rheumatoid arthritis and multiple sclerosis, where TNF-α also contributes importantly to pathogenesis [16, 17]. Additionally, relatively small doses of glucocorticoids (comparable with doses which are effective in rheumatoid arthritis) are sufficient for negative effects on some non-monocytic cells, the most notable effects being suppressed secretion of hypothalamic corticotropin-releasing hormone (CRH) and suppressed expression of the pro-opiomelanocortin (POMC) gene and its product, adrenocorticotropic hormone (ACTH) by the pituitary gland [18]. The result is suppression of the hypothalamic-pituitary-adrenal axis (HPA) axis, with diminished production of endogenous cortisol. Therapeutic concentrations of glucocorticoids are known also to affect at least one monocyte function *in vivo*, that is, the number of monocytes in peripheral blood [19]. A decline in blood monocyte count occurs during conventional therapy. This decline makes it difficult to interpret the glucocorticoid-induced reduction in circulating TNF-α reported in blood of volunteers treated with lipopolysaccharide (LPS) [20].

We therefore administered single doses of prednisolone of 1.5 mg kg^{-1} to healthy volunteers and observed the extent and duration of glucocorticoid-mediated suppression of TNF-α release from peripheral blood monocytes. An *ex vivo* whole blood incubation method was used to avoid the administration of toxic agents (such as LPS) to human volunteers and to avoid the confusing influence of changing monocyte numbers in LPS-treated and prednisolone-treated human subjects. The intensity and duration of monocytopaenia after prednisolone administration provided an additional *in vivo* index of monocyte response and this was compared with the intensity and duration of suppressed plasma cortisol concentration. Finally, glucocorticoids act on many cell types to alter release of products which potentially modify monocyte/macrophage functions. The POMC gene products ACTH [21], β-endorphin [22] and α-melanocyte stimulating hormone (α-MSH) [23] all modulate the activity of monocyte/macrophages experimentally, as do CRH and somatostatin [24, 25]. These are principally products of neuroendocrine tissue, but inflammatory cells have also been shown to release CRH and somatostatin and to express the POMC gene [23, 25]. CRH secretion and POMC gene expression are glucocorticoid-sensitive in neuroendocrine tissue, at least [18], whilst somatostatin release from inflammatory tissue may also be glucocorticoid-sensitive [25]. It is therefore possible that the observed effects of prednisolone on monocyte TNF-α release were not direct, but were secondary to a glucocorticoid-induced change in non-monocytic tissue. This possibility was examined by attempting to reverse the observed suppression of TNF-α release with the glucocorticoid antagonist, mifepristone and by determining whether the relationship between prednisolone concentrations in the blood of the volunteers and TNF-α suppression could be reproduced by adding prednisolone in known concentration to blood taken from the same volunteers when they were drug-free.

Methods

Sampling procedure

A single oral dose of prednisolone of approximately 1.5 mg kg^{-1} was administered to eight healthy, fasting volunteers (20–42 years, two female) as tablets (Fawns & McAllan, Victoria, Australia). Blood samples were collected before dosing and at 2, 4, 8, 12, 24 and 48 h post-dose into EDTA- and heparin-treated evacuated tubes (Venoject™). EDTA-anticoagulated blood was used for automated differential white cell counts by a Coulter STKS Analyser [26] and heparinised blood was used for measurement of plasma cortisol and prednisolone by h.p.l.c. (see below). Separated heparinised plasma was also tested for capacity to suppress monocyte TNF-α production (see below).

Two weeks later, a further 70 ml blood sample was collected into heparinised tubes, pooled and then divided into aliquots of 3.5 ml. Each aliquot was centrifuged and 1.3 ml of plasma was removed and replaced with the same volume of plasma from one of the samples collected in the first 48 h. Each 3.5 ml sample then yielded triplicate samples of 1 ml each in 24 well cell culture plates (Costar). This procedure ensured that equal numbers of autologous monocytes were present with each of the original plasma samples, a condition which could not be achieved using the original, unseparated whole blood samples because of prednisolone-induced changes in monocyte numbers. The concentrations of cortisol and prednisolone in the 1.3 ml of plasma added to the samples had been measured by h.p.l.c. and therefore were known. The original volume of plasma in the samples was calculated from the haematocrit, allowing an estimate of the total prednisolone concentration in the reconstituted samples. The calculation disregards the small amount of free prednisolone which equilibrates with erythrocyte contents [27]. Reconstituted whole blood samples were stimulated with 0.1 μg ml^{-1} LPS (*E. coli*, serotype 026–B6; Sigma), added 30 min after reconstitution. Additional triplets were prepared using plasma from the final sample and were not stimulated with LPS, to indicate baseline, unstimulated TNF-α production. Samples were then incubated for 4 h at 37°C and 5% CO_2, as previously described [28]. Plasma was re-separated by centrifugation and TNF-α was measured by sandwich enzyme-linked immunosorbent assay, as previously described [29, 30]. Recombinant human TNF-α, a gift from Dr G.R. Adolf (Boehringer, Ingelheim), served as standard. Samples were diluted 10 fold in RPMI medium for assay and standards were likewise reconstituted in RPMI medium containing 10% normal human serum. All additives used in the procedure were checked for endotoxin contamination using the Limulus amebocyte lysate test (BioWhittaker Inc, Walkersville, MD) and rejected if endotoxin concentration

exceeded 0.1 u ml^{-1}. All glassware was baked prior to use and all plastic-ware was new.

Additional incubates were prepared from the 4 h specimens (seven subjects) or 8 h specimen (one subject) and were treated with mifepristone (RU 486: Roussel-Uclaf) at 50 μM, to seek evidence that suppression of TNF-α production was due to glucocorticoid present in the sample. This was further investigated by attempting to reproduce the observed relationship between prednisolone concentration and TNF-α suppression by adding prednisolone in known concentration to blood taken from the same volunteers when they were drug-free. A further set of incubates was therefore prepared in the same way from each volunteer's final blood sample, except that the plasma was derived from the same sample (i.e. removed by centrifugation and then returned) and prednisolone (Sigma) was added to concentrations of 1.8×10^4 ng ml^{-1} (50 μM), 1.8×10^3 ng ml^{-1} (5 μM), 360 ng ml^{-1} (1 μM) and 36 ng ml^{-1} (0.1 μM). LPS was introduced 30 min later and incubation proceeded, as above. The relationship between prednisolone concentration and suppression of LPS-stimulated TNF-α release in these samples was compared with the relationship apparent from studies of the samples taken in the 48 h after the dose. Studies on human volunteers were approved by the Human Rights Committee of the University of Western Australia and each participant provided written consent.

Glucocorticoid assays

Prednisolone and cortisol were measured in plasma by h.p.l.c., using a LC5000 pump, an AS2000 automatic injector (both from ICI Instruments, Melbourne, Australia) and a SP1500 integrator (Spectral Physics, CA). The mobile phase was prepared by mixing dichloromethane (AR grade, Merck), ethanol (AR grade, Merck) and water (17 : 1 : 1). The resultant organic phase was separated and 1% glacial acetic acid was added. Flow rate was 1 ml min^{-1}. A Porosil 30 cm × 4 mm column (10 μm; Waters Instruments) was used for normal-phase separation and glucocorticoids were quantitated by absorbance at 254 nm, using a Waters 484 Tunable Absorbance Detector. Dexamethasone (250 ng in ethanol; Sigma) was added to each sample as internal standard. Standards for cortisol and prednisolone (Sigma) were prepared in charcoal-stripped plasma of a normal donor [31]. Samples and standards (1 ml) were extracted sequentially with 8 ml hexane (AR Grade, Merck) and then with 8 ml dichloromethane. The dichloromethane was washed with 1 ml of 0.1 M NaOH followed by 1 ml of distilled water before being separated and evaporation under a nitrogen stream. The resultant extract was dissolved in 100 μl mobile phase and 50 μl was injected. Assay sensitivities were 10 ng ml^{-1} for cortisol and prednisolone.

Data analysis

Data are expressed as mean ± s.e.mean and as 95% confidence intervals (CI) for mean differences, where appropriate. Statistical analysis was by ANOVA (SigmaStat, Jandel Scientific) and multiple comparisons against the pre-dose sample (control) for plasma cortisol, plasma prednisolone, monocyte count and LPS-stimulated TNF-α release were made using Dunnett's test. Prednisolone concentration-time data was fitted to a single compartment pharmacokinetic model incorporating first-order absorption and elimination processes, using a non-linear least-squares fitting procedure (TopFit 2.0) [32].

Results

Prednisolone, cortisol and monocyte kinetics

The highest prednisolone concentration (854 ± 49 ng ml^{-1}) was in the 2 h sample in all volunteers. The mean elimination half-life estimated by pharmacokinetic modelling was 3.15 h (Figure 1). The mean cortisol concentration before dosing was 134 ± 42 ng ml^{-1} and was suppressed by 83% at 2 h. The 95% CI for the mean difference was 46 to 175 ng ml^{-1}. By 8 h, cortisol was unmeasurable in plasma in three of the

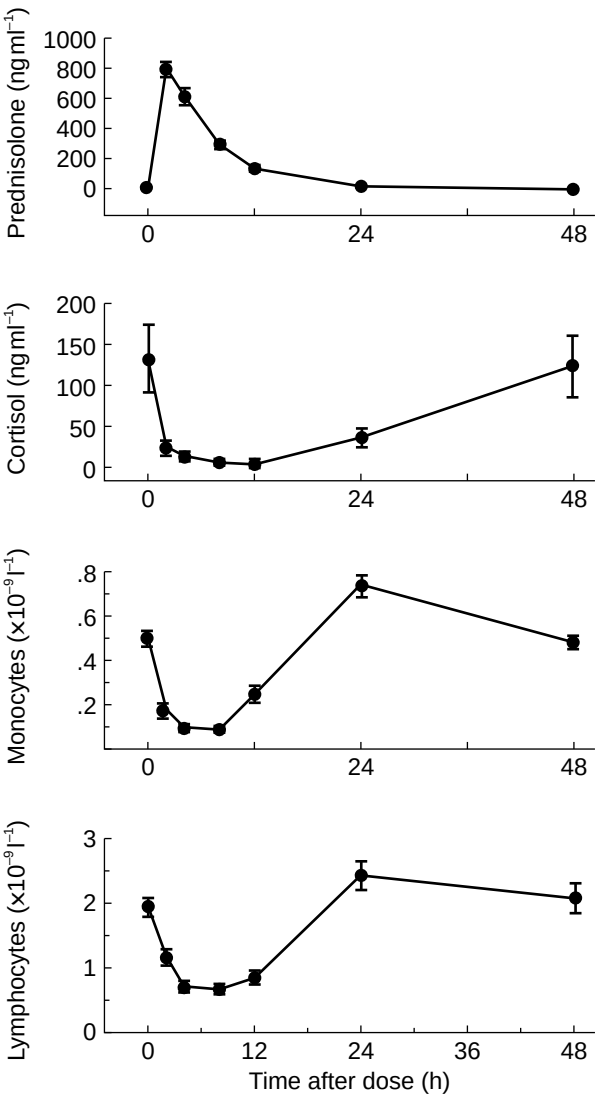

Figure 1 Relationship between prednisolone concentrations, cortisol concentrations and monocyte and lymphocyte numbers in peripheral blood for 48 h after oral administration of 1.5 mg kg^{-1} prednisolone. At each time point from 2 h to 24 h, the plasma cortisol concentration, the number of monocytes and the number of lymphocytes differed significantly ($P < 0.05$; $n = 8$) from pre-dose values.

volunteers (limit of detection: 10 ng ml^{-1}). Cortisol concentrations at 24 h (40 ± 11 ng ml^{-1}) remained significantly lower than pre-dose, despite the absence of measurable prednisolone in five subjects (limit of detection: 10 ng ml^{-1}). The 95% CI for the mean difference was 28 to 168 ng ml^{-1}. Cortisol concentrations recovered to pre-dose levels at 48 h (Figure 1).

The predose blood monocyte count in the volunteers was $0.57 \pm 0.03 \times 10^9$ l^{-1}. Monocyte numbers fell to a mean of 18% of pre-dose levels at 8 h. The 95% CI for the mean difference was 0.34×10^9 l^{-1} to 0.48×10^9 l^{-1}. Partial recovery occurred by 12 h and a rebound monocytosis occurred at 24 h in all volunteers ($P < 0.05$, compared to pre-dose level; 95% CI for the mean difference of 0.15×10^9 l^{-1} to 0.33×10^9 l^{-1}). Monocyte numbers had returned to pre-dose levels at 48 h (Figure 1). Similar variations were seen in lymphocyte numbers in all volunteers (Figure 1). In contrast the number of polymorphonuclear leukocytes significantly exceeded baseline ($3.2 \pm 0.23 \times 10^9$ l^{-1}) at between 2 h and 24 h, with a peak at 4 h of $7.6 \pm 0.67 \times 10^9$ l^{-1}.

Ex vivo *release of TNF-α by whole blood*

Whole blood samples reconstituted with pre-dose plasma released 7.1 ± 0.64 ng ml^{-1} of TNF-α in response to stimulation with LPS for 4 h, whilst unstimulated samples released none. Whole blood samples reconstituted with plasma from subsequent time points to 12 h exhibited lower release of TNF-α after LPS stimulation, in relation to the concentrations of prednisolone therein (Figures 1 and 2). Mean LPS-stimulated TNF-α level was suppressed to 27% (95% CI for the mean difference was 4.3 to 6.1 ng ml^{-1}) and 28% (95% CI for the mean difference was 4.1 to 6.1 ng ml^{-1}) by the 2 h and 4 h plasma samples, respectively. Concentrations of TNF-α in the LPS-stimulated 24 and 48 h samples did not differ significantly from the pre-dose samples. The glucocorticoid antagonist, mifepristone (50 μM) alone did not lead to release of TNF-α from unstimulated blood samples. Rather, it slightly suppressed LPS stimulated release from the final blood sample (2 weeks) from 7.7 ± 0.86 ng ml^{-1} to 7.3 ± 0.77 ng ml^{-1} in the absence of prednisolone ($P = 0.03$, Paired *t*-test). However, treatment of the reconstituted 4 h sample with 50 μM mifepristone restored mean LPS-stimulated TNF-α levels to 72% of the pre-dose sample (seven subjects). The 95% CI for the mean difference between mifepristone-treated and untreated samples was 0.22 to 3.44 ng ml^{-1}. Mifepristone treatment of the 8 h sample from the eighth volunteer restored release to 93%.

Prednisolone, added to whole blood samples collected after the complete elimination of the dose (2 weeks), suppressed TNF-α production in a concentration-dependent manner in each of the volunteers. Measurable TNF-α was not released in the absence of LPS. The concentration of TNF-α in LPS-stimulated samples, in the absence of added prednisolone, was 7.7 ± 0.86 ng ml^{-1}. Although volunteers varied in their response to LPS, the relative suppression of TNF-α release by prednisolone was very similar for all. At 1 μM added prednisolone, mean LPS-stimulated TNF-α production was 27% of production by control samples. The 95% CI for the mean of the difference between prednisolone (1 μM)-treated and control samples was 4.13 to 6.82 ng ml^{-1}. A relationship between the concentration of added prednisolone and TNF-α suppression was apparent. This was very similar to the relationship between measured prednisolone concentrations and suppression of TNF-α release in post-dose plasma samples (Figure 3).

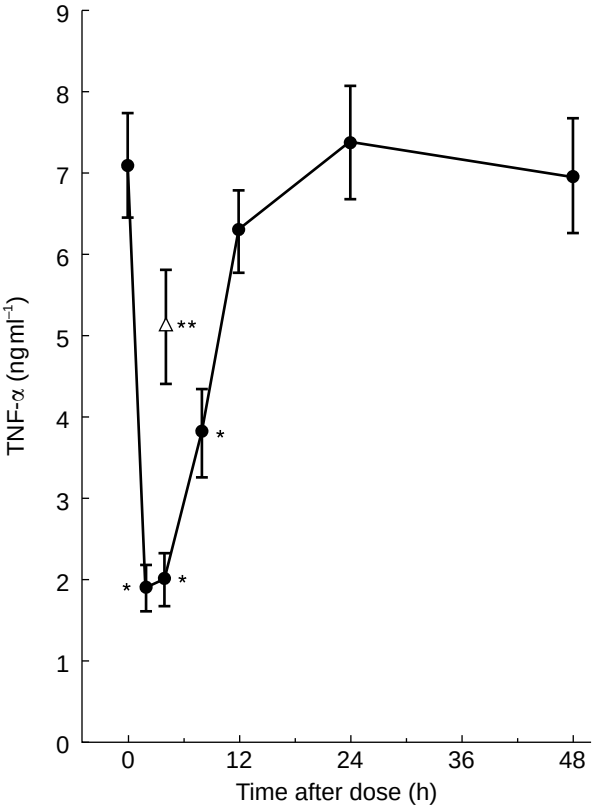

Figure 2 TNF-α concentrations in LPS-stimulated peripheral blood samples reconstituted with plasma which was collected before oral dosing with prednisolone and for 48 h thereafter. △ mifepristone, added at 50 μM to 4 h samples from seven subjects partially reversed suppression of TNF-α. A similar effect was produced in the 8 h sample of the eighth volunteer (result not shown). ⋆$P < 0.05$, compared with pre-dose values ($n = 8$). ⋆⋆$P < 0.05$, compared with 4 h value in absence of mifepristone ($n = 7$).

Additional blood samples were collected from one volunteer before the dose and at 24 h. They were stimulated with LPS immediately after collection and without separation, to find out whether monocytes had recovered capacity for TNF-α release at 24 h after prednisolone exposure *in vivo*. TNF-α release from the pre-dose sample was 7.8 ng ml^{-1}, corresponding to 13 ng 10^6 cells^{-1}, whilst the release at 24 h was 14.1 ng ml^{-1}, corresponding to 17.6 ng 10^6 cells^{-1} and indicating complete recovery.

Discussion

For at least 8 h after a single oral dose of 1.5 mg kg^{-1}, prednisolone is present in plasma in concentrations and forms which are sufficient to suppress release of TNF-α by monocytes. At 24 h, monocyte numbers and capacity for TNF-α release have recovered but hypocortisolaemia persists. Together, these observations imply that prednisolone actions

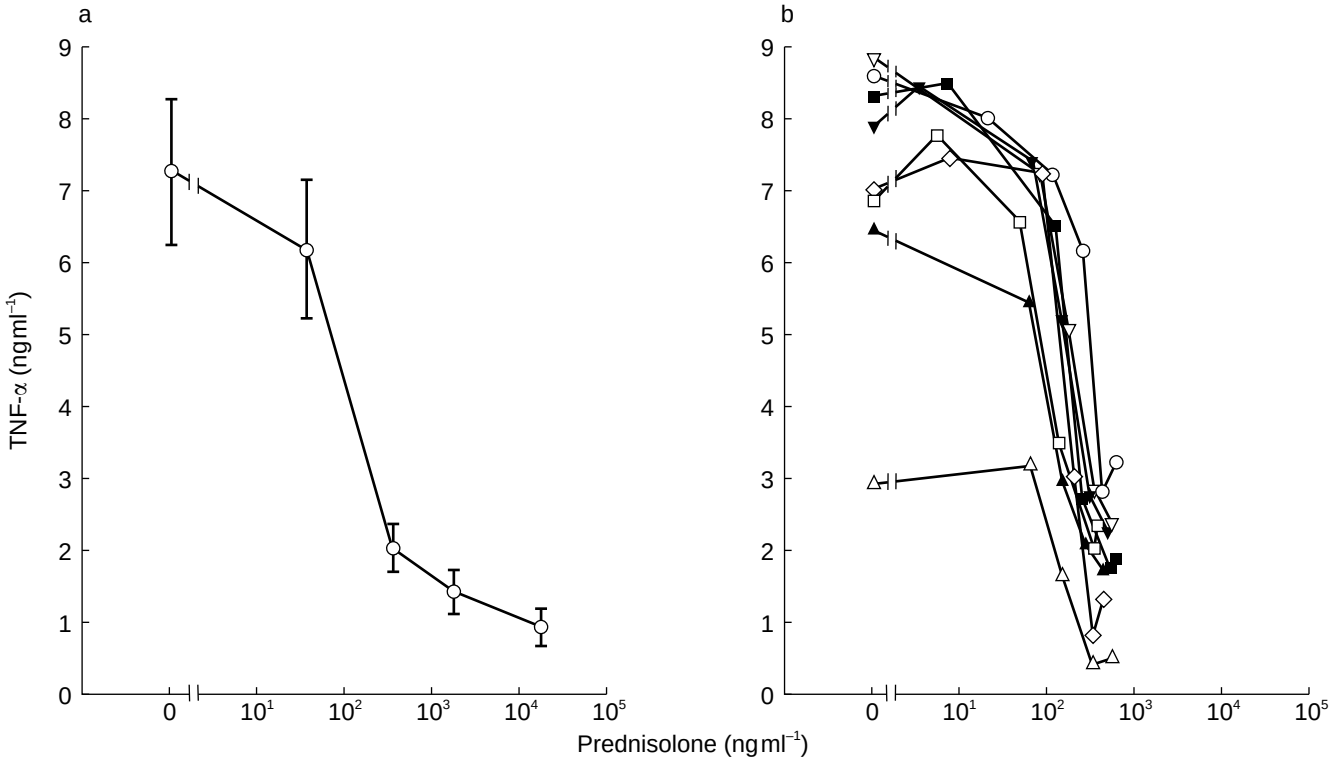

Figure 3 Blood samples were collected from the eight volunteers when they were drug-free. Prednisolone was added to samples at known concentrations and they were stimulated with LPS, as described in methods. The relationship between concentrations of added prednisolone and LPS-stimulated TNF-α in whole blood samples of eight volunteers is shown in (a) (mean ± s.e.mean). This is compared with the relationship between measured prednisolone and TNF-α in whole blood reconstituted with plasma collected after oral dosing with prednisolone in the same subjects (b). The eight subjects are each represented by a different plot. Each point represents the measured prednisolone and TNF-α concentrations at one time point, in one subject. Two samples from each volunteer (pre-dose and 48 h) and 24 h samples from three volunteers contained no measurable prednisolone. The TNF-α values at zero prednisolone concentration are the means of the levels in these samples for each subject.

on blood monocytes are relatively short-lived compared with effects on the HPA axis. Blood monocytes are constantly replenished from precursors in bone marrow possibly contributing to more rapid recovery [33]. Plasma cortisol concentrations recover abruptly after suppression, reflecting the pulsatile nature of secretion [11]. This would have occurred at some time between 24 and 48 h in these volunteers. Differences in the duration of glucocorticoids action on target and other tissues would be important determinants of the balance between efficacy and toxicity.

The partial reversal by mifepristone of TNF-α suppression in 4 h specimens confirms that the action of prednisolone is mostly direct and not through some glucocorticoid-induced modification of other circulating plasma components such as ACTH [21], β-endorphin [22], α-MSH [23], CRH or somatostatin [24, 25]. Mifepristone incompletely reversed the prednisolone effect, possibly because concentrations were insufficient to completely antagonise the high concentrations of prednisolone present at the time, or possibly because mifepristone acts as a partial glucocorticoid agonist under some circumstances [34]. Mifepristone caused a small, but significant, suppression of LPS-stimulated TNF-α release in prednisolone-free samples, suggesting that it acts as a partial glucocorticoid agonist at this relatively high concentration (50 μM) and under these conditions. Mifepristone behaves as a glucocorticoid agonist in respect of the negative regulation of AP-1-mediated transactivation of the collagenase gene, at least [35], and AP-1 appears to be important in the transactivation of the TNF-α gene [36]. The interactions between glucocorticoids, mifepristone, AP-1 and the TNF-α promoter have yet to be investigated. The effects of prednisolone were concentration-dependent and were very similar for orally-administered prednisolone and for prednisolone added after blood was collected. Again, this indicates that the actions of prednisolone are likely to be direct.

Prednisolone induced profound, but transient, monocytopaenia. Glucocorticoids reduce the bone marrow production of monocytes by approximately 20% in mice [19, 37]. However, the mean transit time of monocytes in the human circulation has been estimated as 36 to 104 h [38], so monocytopaenia develops more rapidly than can be explained by cessation of monocyte release from bone marrow alone. The findings of this and previous studies [37] imply that monocytes leave circulating blood at an accelerated rate. The site of redistribution has not been defined. Lymphopaenia and recovery, which occurred synchronously with monocytopaenia and recovery in these studies are understood to result from redistribution of lymphocytes between blood and other body compartments, particularly the bone marrow [39, 40]. Studies in mice have shown that glucocorticoids reduce, rather than increase, the egress of monocytes into the peritoneum, but egress into bone marrow has not been examined [37]. The monocytes may not, in fact, leave the blood. It has been estimated that approximately 75% of monocytes in human blood are

marginated and not circulating [41], so glucocorticoids may act to alter the balance between the circulating and marginated pools in blood. Re-entry of monocytes to the circulation would explain the relative monocytosis at 24 h. The monocytosis also occurred at a time when prednisolone was unmeasurable in most subjects and cortisol concentration had incompletely recovered, so it may reflect relatively low blood glucocorticoid activity at this time. Adherence to vascular endothelium and egress from the circulation involve interactions between adhesion molecules on the surfaces of monocytes and endothelial cells. The glucocorticoid, prednylidine, is reported to increase the adhesion of monocytes to human umbilical vein endothelial cells *in vitro* [42]. To date, however, the described effects of glucocorticoids on adhesion molecules on endothelial cells and monocytes have not suggested a mechanism for glucocorticoid-induced monocytopaenia [33, 42–44]. The relationship between monocytopaenia and the anti-inflammatory action of glucocorticoids remains to be defined.

These studies have therefore confirmed that prednisolone can suppress the release of TNF-α from circulating monocytes *in vivo*. This is consistent with the proposition that glucocorticoids suppress TNF-α release in disorders such as rheumatoid arthritis and multiple sclerosis, where TNF-α is important in pathogenesis [1–3]. *In vitro*, concentrations of glucocorticoids sufficient to suppress TNF-α release also suppress release of IL-1β, IL-6, IL-8 and a range of other monocyte functions, implying that these, too, are modulated *in vivo* by glucocorticoid therapy [6, 7]. The anti-inflammatory and immune suppressant actions of glucocorticoids are mediated by actions on monocyte/macrophages and a range of other cell types [4]. Inflammatory and autoimmune diseases differ in pathogenesis and sensitivity to glucocorticoids, so further research is required to identify the principal therapeutic actions of glucocorticoids in different disorders.

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