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The Chronobiology of Human Cytokine Production

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Cytokine production in human whole blood exhibits diurnal rhythmicity. Peak production of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-1 and IL-12 occurs during the night and early morning at a time when plasma cortisol is lowest. The existence of a causal relationship between plasma cortisol and production is suggested by the finding that elevation of plasma cortisol within the physiological range by the administration of cortisone acetate results in a corresponding fall in pro-inflammatory cytokine production. Cortisol may not be the only neuroendocrine hormone that entrains cytokine rhythms; other candidates include 17-hydroxy progesterone, melatonin and dihydroepiandrostene dione (DHEAS). The finding of diurnal cytokine rhythms may be relevant to understanding why immuno-inflammatory disorders such as rheumatoid arthritis or asthma exhibit night-time or early morning exacerbations and to the optimisation of treatment for these disorders. Diurnal rhythmicity of cytokine production also has implications for the timing of blood samples drawn for diagnostic T-cell assays. Finally, diurnal rhythmicity of immune function suggests that the nature of an immune response, for example in response to vaccination, may be modified by the time of day of antigen administration and raises the possibility that immune responses could be therapeutically manipulated by co-administration of immunoregulatory hormones such as glucocorticoids.

Keywords: Human; Diurnal; Cytokine; Immunity; Rhythms; Hormones

INTRODUCTION

It is increasingly apparent that many important biological functions are subject to diurnal variation. Diurnal rhythms of immunological

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relevance include variation in the number of circulating T cells [1], the autologous mixed lymphocyte reaction [2], the phagocytic index [3] and urinary neopterin secretion [4]. Cytokines play a critical role in mediating and regulating immune effector function. Until recently little was known about diurnal rhythmicity of cytokine production. If human cytokine production was subject to diurnal variation this might account for time-of-day related exacerbations in the symptoms of certain chronic immuno-inflammatory disorders, for example rheumatoid arthritis or asthma. The serum level of many cytokines is below the sensitivity threshold of available assays. Consequently, to address the question of whether human cytokine production is subject to diurnal variation, we applied a whole blood assay [5] to study diurnal changes in antigen or mitogen-stimulated whole blood cytokine production in healthy subjects [6,7]. The advantages of whole blood over purified peripheral blood mononuclear cells (PBMC) are that it can be assayed immediately and, being unmanipulated, most closely approximates the environment *in vivo*. In particular, whole blood contains physiological concentrations of hormones and other circulating factors that regulate T-cell function and could therefore contribute to diurnal variation in cytokine production.

DIURNAL RHYTHMS IN HUMAN CYTOKINE PRODUCTION

Interleukin (IL)-1 and IL-6 are detectable in the serum of healthy subjects only between midnight and 3 am, supporting the hypothesis that cytokine production is subject to diurnal variation [8]. However, most cytokines cannot normally be detected in human plasma or serum. In initial studies with antigen- or mitogen-stimulated whole blood we demonstrated that IFN-γ production to tetanus, phytohaemagglutinin (PHA) or lipopolysaccharide (LPS) exhibited significant diurnal rhythmicity [6]. The IFN-γ rhythm had a similar phase irrespective of the stimulus used. Furthermore, no difference was observed between the rhythms of male and female subjects. Overall, IFN-γ was highest in blood taken between midnight and 3 am and was lowest in blood taken between 8 and 11 am. Interestingly, in one subject, a shift worker, the IFN-γ rhythm was phase advanced by approximately four hours in line with his altered sleep/wake cycle.

We then asked whether other cytokines, IL-1, IL-6, IL-10, IL-12 or TNF-α, also exhibit diurnal rhythmicity. In fact, each of these cytokines was found to be subject to diurnal rhythmicity [7]. As IFN-γ and IL-12 have the ability to upregulate each other's production, not unexpectedly, the rhythms of IFN-γ and IL-12 production were synchronous (Fig. 1(a)). Interestingly, the rhythms of the monokines IL-1, TNF-α and IL-10 (Fig. 1(b)) were also closely synchronous, all peaking at 9 pm, or approximately four hours earlier than the peak of IFN-γ or IL-12.

DIURNAL CYTOKINE RHYTHMS AND Th1/Th2 BALANCE

IFN-γ, a product of T helper 1 (Th1) and natural killer (NK) cells, mediates cellular (type 1) immunity, whereas IL-10 a product of monocytes, Th2 and to a lesser extent Th1 cells, upregulates humoral (type 2) and downregulates type 1 immunity [9,10]. Whether an immune response develops in either a type 1 or type 2 direction depends largely upon the cytokine environment in which T cells are activated. Whereas IFN-γ and IL-12 bias toward a type 1 response, IL-4 and IL-10 bias toward a type 2 response by inhibiting type 1 cytokine production [11–13].

There is a good correlation between the induction of type 2 responses and the expression of IL-10 [14]. This has been shown for S. Mansoni [15] or AIDS infection in mice [16], and HIV infection in humans [17]. Furthermore, the level of IL-10 expression closely correlates with susceptibility of different mouse strains to infection with Candida [18] or Trypanasoma cruzi [19] which depend upon cellular immunity for clearance. Although the role of IL-10 in human immune patho-physiology is not as well defined, the IFN-γ to IL-10 ratio has been found useful in determining the pro- or anti-inflammatory bias of T-cell culture supernatants [20]. We asked, therefore, whether the balance between type 1 and type 2 cytokine production is itself subject to diurnal variation. To address this question, we determined the ratio of IFN-γ to IL-10 production in stimulated whole blood as a possible index of type 1/type 2 immune balance [21]. The IFN-γ/IL-10 ratio exhibited a diurnal rhythm which had a different phase and amplitude to the rhythms of either IFN-γ or

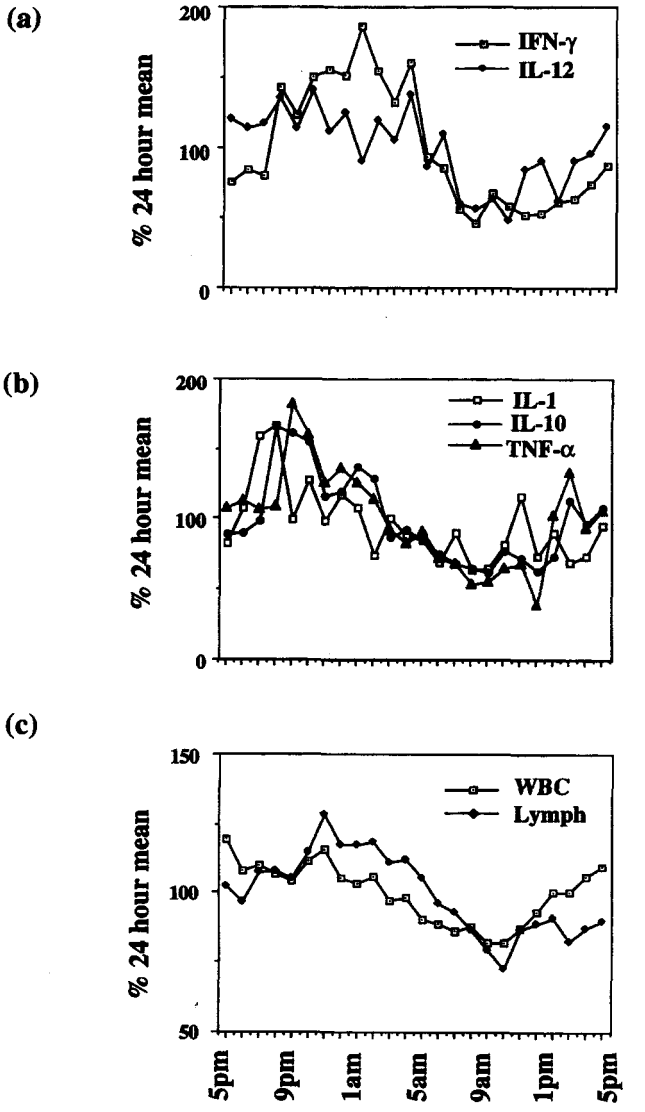

FIGURE 1 **Diurnal rhythms of cytokines and white blood cells in healthy individuals.** LPS-stimulated whole blood production of IFN-γ and IL-12 (a) and IL-1, Il-10 and TNF-α (b). White blood cell (WBC) and lymphocyte (lymph) count in whole blood (c). Results shown are the means of individual subject data ($n = 10$) standardised as a percentage of each subject's 24-hour mean.

IL-10. The IFN-γ/IL-10 ratio peaked at 4 am and reached a nadir at 3 pm. The ratio was negatively correlated with plasma cortisol and its peak was synchronous with the cortisol nadir. Oral administration of cortisone acetate 25 mg markedly reduced the IFN-γ/IL-10 ratio (Fig. 2), suggesting that the diurnal rhythm in the IFN-γ/IL-10 ratio may arise as a consequence of IFN-γ and IL-10 differing in their sensitivity to inhibition by plasma cortisol.

As IFN-γ and IL-10 are markers of cellular and humoral immunity respectively, the above findings suggest there is a bias toward cellular immunity during the night and early morning when the IFN-γ/IL-10 ratio is high. Conversely, there may be a relative bias away from cellular immunity during the day. These findings are consistent with the fact that symptoms of chronic inflammatory disorders, for example rheumatoid arthritis [22] or asthma [23], are most severe during the night and early morning.

We speculate that changes in the IFN-γ/IL-10 ratio reflect an underlying diurnal rhythm in type1/type2 balance with alternating periods of cellular or humoral dominance within any 24-hour period. This rhythm could be important in the regulation of cellular and humoral balance during primary immune responses. Type 1 and type 2 immune responses exhibit reciprocal antagonism; IFN-γ inhibits Th2 cells whereas IL-4 and IL-10 inhibit Th1 cells. Alternating periods of type 1 and type 2 bias may help facilitate the parallel development of otherwise mutually antagonistic arms of the immune response. As the primary immune response matures, one or other response may preferentially expand and ultimately override this alternating diurnally-imposed bias, thereby resulting in either type 1 or type 2 polarisation of the immune response.

We would speculate further that the diurnal rhythm of type 1/type 2 balance arose in response to evolutionary pressures. Cellular responses mediate delayed-type hypersensitivity inflammation associated with swelling, pain, immobility and malaise. Therefore, it would be advantageous to bias type 1 responses to inactive 'healing' periods (night-time in humans) and not to active periods when maximum mobility is required for hunting, gathering and 'fight or flight' reactions. In molecular terms, this could be effected, for example, by differential responsivity of the promoters of type 1 and type 2

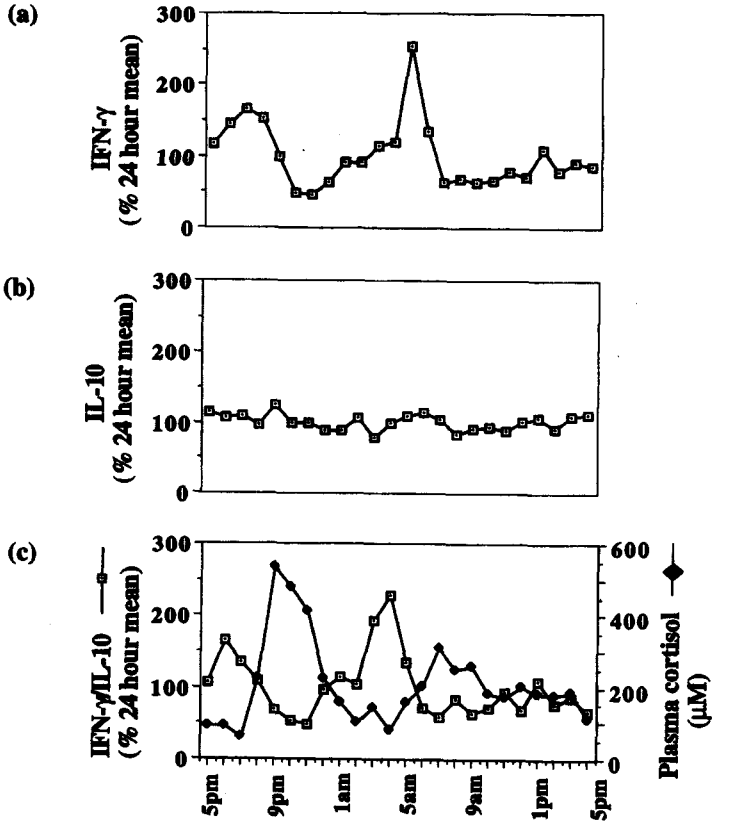

FIGURE 2 **Effect of administration of cortisone acetate, 25 mg at 9 pm, on LPS-stimulated whole blood production of IFN-γ and IL-10, and the IFN-γ/IL-10 ratio.** Results shown are the means of individual subject data ($n = 5$) standardised as a percentage of each subject's 24-hour mean. Mean plasma cortisol levels in response to cortisone administration are shown superimposed on the IFN-γ/IL-10 ratio (c).

cytokine genes to positive or negative regulation by glucocorticoids or other immunomodulatory hormones, the latter being entrained to the day–night cycle via the retinal–pineal–melatonin axis.

MECHANISMS UNDERLYING DIURNAL CYTOKINE RHYTHMS

The mechanisms of immune system bio-rhythmicity are not well defined, but are presumed to be neuroendocrine-based, in large part.

A variety of neuro-endocrine hormones exhibit both diurnal rhythmicity and immuno-modulatory actions; examples include cortisol, melatonin, 17-hydroxy progesterone, dihydroepiandrostenedione sulphate (DHEAS), growth hormone (GH), prolactin, vasopressin and β-endorphin [24].

Cortisol, the major circulating human glucocorticoid, is a powerful natural immuno-suppressant. Plasma cortisol exhibits a well-defined diurnal rhythm [25] which could be anticipated to impose diurnal variation on immune responsiveness. This being the case, periods of heightened immune reactivity would be anticipated to coincide with or follow the early morning nadir in plasma cortisol. We found that IFN-γ, IL-12, TNF-α and, to a lesser extent, IL-10 production in response to tetanus or LPS was inversely correlated with plasma cortisol [7]. Production of these cytokines tends to be highest in blood taken during the late evening or early morning, at a time when the plasma cortisol is low. In support of a causal relationship between high plasma cortisol and low IFN-γ production, the reduction in IFN-γ production that occurs in association with the peak of endogenous cortisol was mimicked by hydrocortisone added to whole blood cultures at concentrations equivalent to high physiological plasma cortisol levels [6]. However, until recently it was not known whether variation of cortisol within the physiological range, as occurs diurnally, modulates cytokine production. We found that elevation of plasma cortisol within the physiological range, by the ingestion of cortisone acetate, significantly reduced tetanus- or LPS-stimulated IFN-γ, TNF-α, IL-1 and IL-12 production, but had little or no effect on IL-10 production [7] (Fig. 2). This argues strongly that diurnal rhythms of pro-inflammatory cytokine production are negatively entrained by the diurnal rhythm of plasma cortisol.

Diurnal rhythms in cytokine production need not be due to a direct effect of cortisol on cytokine synthesis but could also reflect time-of-day dependent or cortisol-mediated changes in the number of circulating cytokine-producing cells. Circulating white cells, in particular CD4+ T cells, peak during the night and early morning, coincident with the nadir in plasma cortisol (Fig. 1(c)) [1,26–28]. This approximates the time of maximal IFN-γ, TNF-α, IL-1, IL-10 and IL-12 production, suggesting a possible causal link. However, we

found that IFN-γ, TNF-α and IL-12 production fell one hour after cortisone acetate administration whereas significant changes in white cell numbers were not evident until 2–3 hours later [7]. It seems unlikely, therefore, that cortisol suppresses cytokine production by suppressing circulating white cell numbers *per se*.

Cortisol downregulates cytokine gene expression by binding to and activating negative regulatory elements in the promoters of cytokine genes [29–31] and by inducing IκBa, a cytosolic protein which binds and neutralises the cytokine transcription factor NF-κB [32]. Consequently, plasma cortisol could reduce whole blood cytokine production by directly downregulating cytokine gene transcription. Cytokines whose transcription has been reported to be downregulated by glucocorticoids include the proinflammatory cytokines IL-2 and IFN-γ [29], IL-6 [31], IL-3, GM-CSF and TNF-α [33] and the chemokine, IL-8 [30]. IL-10 has anti-inflammatory actions [14] which may explain why we found it to be relatively resistant to cortisol suppression. We propose that during the night and early morning low plasma cortisol permits maximal IFN-γ transcription resulting in a high IFN-γ/IL10 ratio and a bias toward cellular immunity. Conversely, during late morning and early afternoon high plasma cortisol suppresses IFN-γ transcription while sparing IL-10 production, thereby resulting in a low IFN-γ/IL10 ratio and a bias away from cellular immunity.

Cortisol, of course, is not the only neuroendocrine factor which could entrain diurnal rhythmicity in immune function. Melatonin, growth hormone (GH), prolactin, 17-hydroxy progesterone and DHEAS also possess immunomodulatory actions [24] and, as we have shown (data unpublished) exhibit diurnal secretion (Fig. 3). Plasma levels of melatonin and androstenedione peak at approximately 3 am, whereas levels of GH and prolactin peak soon after the onset of sleep [34]. Levels of 17-hydroxyprogesterone and cortisol both peak at approximately 9 am. Melatonin stimulates IL-1 [35] and IFN-γ [36] production by human macrophages and mouse splenocytes, respectively, and counteracts the immunosuppressive effects of glucocorticoids on antiviral resistance and thymic weight in mice [37]. Similarly, DHEAS has been reported to bias towards type 1 cytokine production [38,39]. GH activates human macrophages and

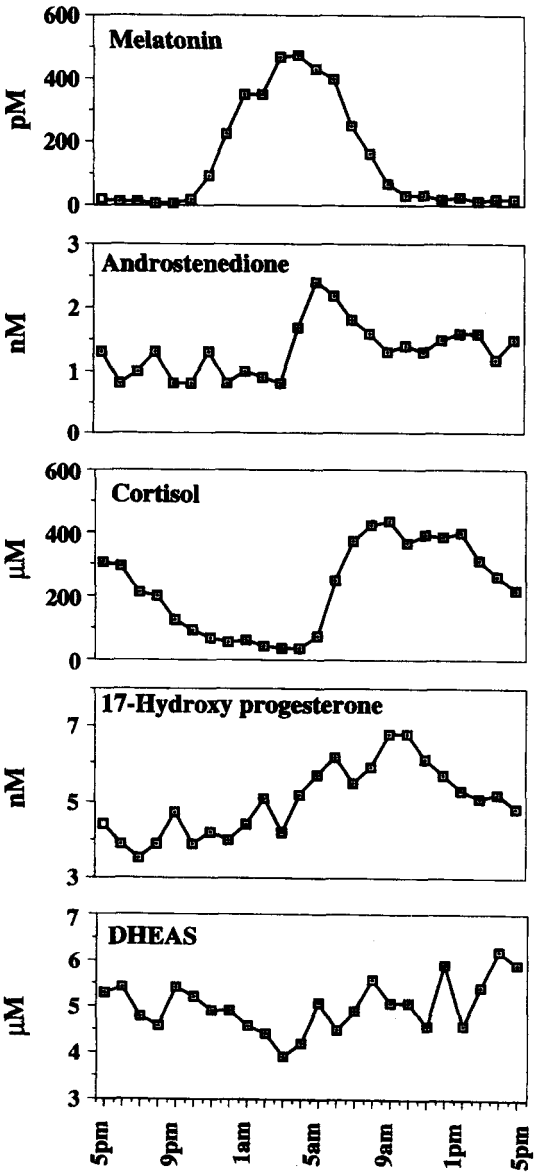

FIGURE 3 **Diurnal rhythms of plasma melatonin, androstenedione, cortisol, 17-hydroxyprogesterone and DHEAS.** Results shown are the data of one representative subject.

primes them for enhanced H_2O_2 release [40] and when given to hypopituitary animals augments antibody synthesis and skin graft rejection [41,42]. Prolactin, likewise, has been shown to enhance immune function [43]. To date, we have not detected any clear relationships between IFN-γ production and plasma GH or prolactin levels. Although there is a positive correlation between whole blood IFN-γ production and plasma melatonin or androstenedione and a negative correlation between IFN-γ and plasma cortisol or 17-hydroxy progesterone thus far, with the exception of cortisol, we cannot say if these hormones independently regulate cytokine expression *in vivo*. It is interesting to note that the diurnal rhythms of cortisol and 17-hydroxyprogesterone, hormones which impart Th2 bias, peak synchronously at approximately 9 am. Likewise, the rhythms of melatonin and androstenedione, hormones which may be associated with Th1 bias, peak synchronously between 3 and 5 am (Fig. 3). The synchronous nature of these rhythms is in keeping with our hypothesis that there is a hormonally-mediated bias towards type 1 responses during the night and early morning and a bias towards type 2 responses during the rest of the day.

DIURNAL CYTOKINE RHYTHMS AND DISEASE

The symptoms of immuno-inflammatory disorders, for example rheumatoid arthritis or asthma, commonly exhibit diurnal rhythmicity. Joint inflammation in rheumatoid arthritis is at its most severe in the early morning [22] and asthma exacerbations commonly occur during the night [23,43]. Impaired function of the hypothalamic-pituitary-adrenal (HPA) axis has been implicated in predisposing to rheumatoid arthritis [44] and nocturnal exacerbations of asthma have been related to increased immune reactivity associated with the early morning nadir in plasma cortisol [45]. It is possible, therefore, that night-time or early morning exacerbations of immuno-inflammatory disorders such as rheumatoid arthritis or asthma reflect diurnally increased production of pro-inflammatory cytokines.

IFN-γ, TNF-α and IL-1 are major inflammatory mediators; TNF-α and IL-1, in particular, play an important role in the pathogenesis of rheumatoid arthritis. Glucocorticoids are highly effective in relieving the symptoms of rheumatoid arthritis but potential side-effects

limit their therapeutic applications. Recently, there has been a resurgence of interest in the use of glucocorticoids because of evidence that low dose therapy reduces joint destruction, in addition to acutely relieving inflammatory symptoms [46]. Glucocorticoids are generally administered as a single and/or major morning dose, the rationale being that administration in phase with the normal diurnal cortisol rhythm may cause less adrenal suppression than multiple daily doses [47]. Although widely used, this schedule might not always be therapeutically optimal. In the treatment of inflammatory conditions with nocturnal exacerbations, it would be advantageous if the peak anti-inflammatory effect occurred during the night. If glucocorticoids are given in the morning, effective nocturnal levels can only be achieved with high doses or long-acting preparations, thereby increasing the likelihood of side-effects. However, the potential exists to use short-acting glucocorticoids in a single evening dose to suppress the night-time increase in pro-inflammatory cytokine production [7].

OTHER IMPLICATIONS OF DIURNAL CYTOKINE RHYTHMS

In vitro assays of T-cell proliferation in response to non-specific stimuli (e.g. lectins) or specific antigens (e.g. tetanus, tuberculin) are used to assess cellular immune function. However, these assays are performed under non-physiological conditions and have poor reproducibility. Recently, assessment of T-cell function has been refined by the measurement of secreted cytokines (interferons, interleukins), and this technique has been applied to the development of diagnostic tests; for tuberculosis in cattle [48] and for immunity to tuberculosis [D. Jones, personal communication], Francisella tularensis [49], leprosy [50] and cutaneous Leishmaniasis [51] in humans. In view of the diurnal nature of cytokine production, inter-assay variation in peripheral blood T-cell cytokine responses will be minimised if blood is drawn at a fixed time of day. Ideally, for maximal responsiveness of pro-inflammatory cytokines, blood would need to be drawn in the late evening or early morning hours. Furthermore, in view of the causal relationship between high plasma cortisol and low pro-inflammatory cytokine production, the effect on cytokine production

of an acute increase in plasma cortisol, for example induced by anxiety in anticipation of venesection, may also need to be considered.

Our findings may also be relevant to other observations of diurnal rhythmicity of immune parameters. IFN-γ has an important role in NK cell activation and increased nighttime IFN-γ production could, therefore, explain the reported increase of NK cell function in human nighttime blood samples [52]. Finally, to reiterate, the direction of an immune response, for example to vaccination, could be modified by the time of day of antigen presentation, raising the possibility that immune responses could be therapeutically manipulated by co-administration of immuno-regulatory hormones. The study of immune function under more physiological conditions in 'free living' humans has important clinical, diagnostic and therapeutic implications, as exemplified by our observations on the diurnal rhythmicity of whole blood cytokine production.

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