Natural killer cell activity in the peripheral blood of patients with Cushing's syndrome

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Abstract

Background: Natural killer (NK) cells are CD3⁺CD16⁺CD56⁺ bone-marrow-derived lymphocytes mediating first-line defence by direct cytotoxicity against various types of target cells without prior immunization. NK cell activity is positively regulated by immune interferon (IFN- γ); among hormones, glucocorticoids are potent *in vitro* and *in vivo* inhibitors, whereas ACTH and β -endorphin in many experimental circumstances enhance NK cytotoxicity.

Design: We measured NK cytotoxicity of peripheral blood mononuclear cells (PBMC) obtained at 0800 h and 2000 h from 26 patients with Cushing's syndrome (12 pituitary-dependent, 12 adrenal-dependent and two dependent on ectopic ACTH secretion). *In vitro* responsiveness to IFN- γ or cortisol was also tested.

Methods: NK activity was measured in a 4-h direct cytotoxicity assay using K562 cells as targets. Plasma ACTH, serum and urinary free cortisol were concomitantly measured with commercially available kits.

Results: Spontaneous activity and responsiveness to IFN- γ or cortisol were significantly greater in 15 age- and sex-matched controls than in Cushing's patients at 0800 h. In pituitary-dependent Cushing's patients, plasma ACTH correlated positively with mean levels of spontaneous NK activity ($r=0.64$, $P<0.05$) and negatively with cortisol-dependent percentage inhibition ($r=-0.69$, $P<0.02$). In adrenal-dependent Cushing's patients, a negative correlation was observed between levels of spontaneous NK activity and urinary free cortisol ($r=-0.67$, $P<0.02$).

Conclusions: Our data indicate that excess endogenous glucocorticoids affect spontaneous NK cell activity and responsiveness to exogenous IFN- γ or cortisol. The differential patterns observed between pituitary-dependent and adrenal-dependent groups are compatible with a positive immunomodulatory role of pituitary pro-opiomelanocortin-derived peptides that effectively counterbalance, at least partially, glucocorticoid immunosuppression.

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Introduction

Natural killer (NK) cells are a subset of bone-marrow-derived lymphocytes with large granular morphology and phenotypically characterized as coexpressing the cell surface antigens CD16 and CD56, and lacking the CD3/T cell receptor complex (1, 2). These cells mediate first-line defence by direct cytotoxicity, without prior immunization, against various types of target cells that lack or down-regulate major histocompatibility complex class I molecules and thereby escape detection by cytotoxic T lymphocytes (3).

Most components of the biology of NK cells are regulated by interleukins (IL) such as IL-1, IL-2, IL-10 and, notably, IL-12, which has the ability to induce the synthesis of immune interferon (IFN- γ) by NK cells themselves. IFN- γ is, indeed, a key molecule for phenotypic differentiation and functional activation of

these cells (4, 5). Hormones are also known to be important regulators of natural cytotoxicity. Glucocorticoids are potent *in vitro* and *in vivo* inhibitors of NK cell activity (6–9), through the mediation of classic glucocorticoid receptors and the reduction of cytosolic calcium availability (10). In contrast to glucocorticoids, two pro-opiomelanocortin (POMC)-derived peptides, adrenocorticotrophic hormone (ACTH) and β -endorphin, have been found to be positively influential upon NK cytotoxicity (9, 11, 12). We demonstrated that both ACTH and β -endorphin enhance the cytokine-induced NK activity and reduce the cortisol inhibitory effect (9, 12).

An interesting feature of NK cell activity is its circadian organization. Both the spontaneous cytotoxicity and the *in vitro* susceptibility to cortisol and IFN- γ oscillate throughout the 24-h cycle (13, 14). Previous work by our group demonstrated that

spontaneous NK cell activity and IFN- γ -dependent boosting of cytotoxicity peak early in the morning, reaching lower levels in late afternoon. In contrast, glucocorticoid-dependent inhibition was observed at its maximum in late evening or night. The circadian variations of NK cytotoxicity are conceivably connected with rhythmic changes in soluble regulatory factors, including hypothalamo–pituitary–adrenal (HPA) hormones among others (15, 16).

The clinical relevance of increased susceptibility to bacterial and mycotic infections in Cushing's syndrome is obvious (17, 18), yet specific immune functions in such patients have been poorly investigated and to the best of our knowledge no systematic study of NK cell function and responsiveness to physiological modifiers has been reported.

We have focused on NK activity of peripheral blood mononuclear cells (PBMC) in patients with Cushing's syndrome, either dependent on excess ACTH (Cushing's disease or ectopic secretion) or having an autonomous adrenocortical hypersecretion. Two opposite circadian stages were considered for both spontaneous cytotoxicity and *in vitro* responsiveness to positive (IFN- γ) or negative (cortisol) physiological modifiers.

Subjects and methods

Twenty-six patients with Cushing's syndrome, 12 pituitary-dependent, 12 adrenal-dependent and two ectopic ACTH-syndromes (3 men and 21 women, age

range 22–60 years) were enrolled in the study (Table 1). All patients had active disease and had not undergone previous treatment. The diagnosis of Cushing's syndrome was based on standard criteria. As controls, 15 healthy normal volunteers (3 men and 12 women, age range 24–58 years) were recruited from the laboratory staff. Neither patients nor controls were receiving medication or displayed any clinical sign of infection.

All controls and patients participated in the study after giving their informed consent. Procedures followed were in accordance with the ethical methods of the institutional responsible committee on human experimentation. Consumption of ethanol and smoking were not allowed in the last 72 h before blood was drawn. Between 0800 h and 2000 h, participants were kept in a quiet environment and allowed to eat, sleep and nap *ad libitum*.

Heparinized blood samples were drawn from patients and controls at two opposing times of the day (0800 h and 2000 h) and PBMC were immediately separated. Before the NK cytotoxicity assay, effector cells were incubated for 20 h in the presence or absence of modifiers: recombinant IFN- γ (rIFN- γ) or cortisol. In addition, blood samples were concomitantly collected from patients for plasma ACTH and serum measurement of cortisol.

Preparation of PBMC and NK activity assay

PBMC were isolated from heparinized peripheral blood by Ficoll-Paque (Cederlane, Ontario, Canada)

Table 1 Clinical and hormonal characteristics of patients with Cushing's syndrome.

Patient no.	Sex	Age (yr)	Duration (yr)/ histology	ACTH (pg/ml)		Cortisol (μ g/dl)		Urinary free cortisol (μ g/24 h)
				0800 h	2000 h	0800 h	2000 h	
1	F	56	2 / pa	14	14	16	25	177
2	F	47	3 / pa	17	17	18	13	111
3	F	58	3 / pa	68	66	17	16	323
4	F	25	4 / pa	18	21	20	17	700
5	F	48	1 / pa	34	38	19	16	288
6	F	60	2 / pa	58	66	18	26	152
7	F	55	5 / pa	58	41	11	4	282
8	F	43	2 / pa	18	15	13	9	153
9	F	24	1 / pa	98	79	17	13	146
10	F	60	4 / pa	82	88	16	20	300
11	M	35	3 / pa	38	35	18	14	184
12	M	19	1 / pa	42	60	18	26	140
13	M	44	2 / aa	<5	<5	29	25	238
14	F	46	1 / aa	<5	<5	45	41	730
15	M	50	2 / aa	<5	<5	55	33	1800
16	F	28	1 / aa	<5	<5	23	21	357
17	F	45	1 / aa	<5	<5	19	18	270
18	F	22	2 / aa	<5	<5	27	30	465
19	M	49	2 / aa	<5	<5	45	50	450
20	F	38	0.5 / ac	<5	<5	23	20	297
21	M	50	0.5 / ac	<5	<5	8	10	302
22	F	42	1 / ac	<5	<5	25	24	434
23	M	49	1 / ac	<5	<5	23	21	510
24	F	21	0.5 / ac	<5	<5	50	38	500

pa, Pituitary adenoma; aa, adrenal adenoma; ac, adrenal carcinoma.

gradient centrifugation (19). After separation, PBMC preparations were washed three times and resuspended in complete medium (RPMI 1640 medium enriched with 10% heat-inactivated fetal bovine serum, 1% glutamine and 50 µg/ml gentamicin). An aliquot (3×10^6 cells/ml complete medium) of PBMC preparations from five patients randomly taken from the two major groups of patients and from five healthy controls was incubated overnight in culture flasks (37 °C, 95% air, 5% CO₂) to remove adherent cells/monocytes. Non-adherent PBMC were centrifuged and resuspended in complete medium at 5×10^6 cells/ml complete medium and incubated with the specific monoclonal antibody anti-CD56 (Becton Dickinson, Lincoln Park, NJ, USA) for 20 min at 4 °C, because anti-CD56 recognizes more than 95% of NK cells (2). After incubation, NK cells were counted by cell sorting.

PBMC preparations (7.5×10^6 /ml) were then exposed to complete medium containing modifiers or alone. Cortisol (Sigma, St Louis, MO, USA) was initially dissolved in 95% ethanol and then diluted in distilled water to give a 1 mmol/l stock solution, which was stored at 4 °C. For use in experiments, the steroid was promptly diluted in complete medium to a final concentration of 10^{-6} mol/l. Ethanol at the final concentration added to effectors (ranging from 100 to 1 µg/ml) did not alter the cell viability or NK activity of our preparations. rIFN-γ (Sigma) was diluted in complete medium and used at the concentration of 650 IU/ml. Incubation at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ lasted 20 h.

Preliminary experiments on the dose–response curve showed that these concentrations of modifiers (cortisol or rIFN-γ) and this incubation time, give the optimal inhibition or enhancement respectively, of NK activity (8, 10, 12).

After incubation, NK assay was performed using a non-radiometric technique reported in detail elsewhere (20). K562 cells from a human chronic myelogenous leukaemia cell line were used as targets. NK activity was expressed in lytic units (LU)/ 10^7 PBMC, according to the equation proposed by Pross *et al.* (21). When the effects of positive or negative modifiers were assessed, data were expressed as percentage changes from control

values obtained under the same conditions in the absence of modifiers (spontaneous activity) (20).

Hormone assays

Serum and urinary free cortisol were determined with a specific RIA method purchased from Sorin Biomedica (Saluggia, Italy). ACTH was assayed by a specific IRMA method purchased from Nichols Institute (San Juan Capistrano, CA, USA). All hormone assays were performed in the same laboratory. Intra- and interassay coefficients of variation for the two above-mentioned variables were less than 8% and 12% respectively.

Statistics

All results are presented as means $\pm$ S.E., except for the two ectopic ACTH syndromes. For all participants, data on the spontaneous NK activity were expressed as absolute values (LU/ 10^7 PBMC), whereas levels of cytotoxicity after incubation of effector cells with modifiers were expressed as percentage changes from spontaneous activity. Because of the skewed distribution, basal values of cytotoxicity or changes from spontaneous activity in controls and pituitary-dependent or adrenal-dependent patients were compared using the Wilcoxon rank test or the Mann–Whitney test where appropriate. When correlations were sought between immune parameters and ACTH or cortisol concentrations, Spearman's rank correlation coefficient was calculated. Outliers were not eliminated from the data sets. In all circumstances, $P=0.05$ or less was regarded as significant.

Results

Plasma ACTH concentration was greater in pituitary-dependent patients (Table 2). Serum and urinary free cortisol concentrations were significantly greater in adrenal-dependent patients than in pituitary-dependent ones.

In the two patients affected with ectopic ACTH secretion, ACTH plasma concentrations were much greater than in pituitary-dependent patients; both serum and urinary free cortisol concentrations were

Table 2 Hormonal patterns in patients with Cushing's syndrome. Values are means $\pm$ S.E.M.

	Plasma ACTH (pg/ml)		Serum cortisol (µg/dl)		Urinary free cortisol (µg/24 h)
	0800 h	2000 h	0800 h	2000 h	
Pituitary-dependent (n = 12)	44.5 $\pm$ 8.3	45.0 $\pm$ 7.5	16.7 $\pm$ 0.7	16.6 $\pm$ 1.9	246.3 $\pm$ 46.4
Adrenal-dependent (n = 12)	< 5	< 5	31.0 $\pm$ 4.1**	27.6 $\pm$ 3.2**	529.4 $\pm$ 121.9**
Ectopic ACTH					
Patient 1	196	194	> 80	> 80	1500
Patient 2	640	520	> 80	> 80	1090

** $P < 0.01$ compared with pituitary-dependent group.

Table 3 Mean $\pm$ S.E. levels of cytotoxicity (absolute values, LU/10⁷ PBMC) obtained after *in vitro* incubation of PBMC in the presence or absence of modifiers, and mean numbers of CD56⁺ cells at 0800 h (percentage values).

	Controls (n = 15)	Pituitary-dependent (n = 12)	Adrenal-dependent (n = 12)
0800 h			
Spontaneous	16.2 $\pm$ 1.4 ^{†††}	10.9 $\pm$ 2.1	6.0 $\pm$ 1.0
+ rIFN- γ (650 IU/ml)	29.5 $\pm$ 2.4 ^{***†††}	14.7 $\pm$ 3.8 ^{***}	11.2 $\pm$ 1.9 ^{***}
+ cortisol (10 ⁻⁶ mol/l)	8.7 $\pm$ 1.0 ^{***†††}	7.3 $\pm$ 1.5 ^{***}	5.3 $\pm$ 0.9 ^{***}
CD 56 ⁺ cells (n = 5)	15.3 $\pm$ 1.5	14.9 $\pm$ 1.5	14.6 $\pm$ 1.4
2000 h			
Spontaneous	6.4 $\pm$ 1.7	11.6 $\pm$ 2.5	9.0 $\pm$ 1.7
+ rIFN- γ (650 IU/ml)	9.7 $\pm$ 1.3 ^{***}	17.7 $\pm$ 3.7 ^{***}	11.2 $\pm$ 2.0 ^{***}
+ cortisol (10 ⁻⁶ mol/l)	2.0 $\pm$ 0.4 ^{***}	7.7 $\pm$ 1.7 ^{***}	6.7 $\pm$ 1.1 ^{**}

P* < 0.01, *P* < 0.001 compared with spontaneous; †††*P* < 0.001 compared with 2000 h.

much greater and superimposable, respectively, with those registered for adrenal-dependent Cushing's patients.

Both in controls and in patients, the levels of spontaneous NK cell activity showed wide interindividual variability.

Mean spontaneous cytotoxicity at 0800 h was greater in controls; statistical significance was attained in the comparison between controls and both groups of patients (*P* < 0.05 compared with the pituitary-dependent group; *P* < 0.01 compared with the adrenal-dependent group). However, no significant differences between controls and patients were observed for the percentage of CD56⁺ positive cells (NK cells) (Table 3). The two patients with ectopic ACTH secretion displayed lower levels of cytotoxicity than healthy subjects (Fig. 1; Tables 3, 4). In the second circadian stage (2000 h),

values were similar in controls and all patients (Fig. 1; Tables 3, 4).

In agreement with previous observations (13, 14), healthy subjects displayed significantly greater levels of spontaneous NK activity at 0800 h than at 2000 h (*P* < 0.01). No appreciable differences between the two time points were observed in Cushing's patients.

In the morning, the percentage rIFN- γ -induced boosting of NK activity was significantly greater in controls than in patients with pituitary-dependent (*P* < 0.001) or adrenal-dependent (*P* < 0.001) Cushing's syndrome; at 2000 h, statistical significance was reached only between controls and the latter group of patients (*P* < 0.01) (Fig. 2; Table 3). The percentage rIFN- γ -dependent enhancement of cytotoxicity in ectopic ACTH secretion is shown in Table 4.

In healthy subjects, rIFN- γ -dependent enhancement of cytotoxicity was significantly greater at 0800 h than at 2000 h, both as absolute (*P* < 0.001) and percentage

Figure 1 Spontaneous NK cell activity of PBMC preparations obtained at two opposing circadian stages (0800 and 2000 h) from healthy subjects (controls) and patients with Cushing's syndrome, either pituitary-dependent or adrenal-dependent. Data are expressed as absolute values (mean $\pm$ S.E.). ***P* < 0.01 compared with values obtained at 2000 h.

Figure 2 *In vitro* responsiveness to rIFN- γ (650 IU/ml) of PBMC preparations obtained at two opposing circadian stages (0800 and 2000 h) from healthy subjects (controls) and patients with Cushing's syndrome, either pituitary-dependent or adrenal-dependent. Data are expressed as percentage changes (mean $\pm$ S.E.) from spontaneous activity of PBMC preparations taken as 100. ***P* < 0.01 compared with values obtained at 2000 h.

Table 4 Levels of cytotoxicity (absolute values expressed as LU/10⁷ PBMC, and percentage values) obtained after *in vitro* incubation of PBMC in the presence or absence of modifiers in two patients with ectopic ACTH secretion.

	Patient 1		Patient 2	
	Cytotoxicity (LU/10 ⁷ cells)	Change from spontaneous activity (%)	Cytotoxicity (LU/10 ⁷ cells)	Change from spontaneous activity (%)
0800 h				
Spontaneous	5.1		2.8	
+ rIFN- γ (650 IU/ml)	8.6	+68.6	4.6	+64.2
+ cortisol (10 ⁻⁶ mol/l)	4.8	-5.8	2.3	-17.8
2000 h				
Spontaneous	6.0		4.0	
+ rIFN- γ (650 IU/ml)	9.1	+51.6	7.3	+82.5
+ cortisol (10 ⁻⁶ mol/l)	4.9	-18.3	3.4	-15.0

values ($P < 0.01$), whereas time-dependent differences were not appreciable in Cushing's patients (Fig. 2; Table 3).

The percentage cortisol-dependent inhibition was always less in patients than in healthy subjects. At 0800 h, patients with adrenal-dependent glucocorticoid excess showed the lowest levels of inhibition, with significant differences from both controls ($P < 0.01$) and pituitary-dependent Cushing's patients ($P < 0.01$) (Fig. 3; Table 3). At 2000 h, controls displayed the highest degree of percentage inhibition, whereas the lowest was again observed in patients with adrenal-dependent Cushing's syndrome (Fig. 3). In the two patients affected by ectopic ACTH secretion, cortisol-dependent inhibition was even lower than in patients with adrenal-dependent glucocorticoid excess (Table 4).

In all patients, levels of cortisol-mediated inhibition recorded at 0800 h and at 2000 h were not significantly different. In contrast, in controls, steroid-dependent

reduction of cytotoxicity was significantly greater at 2000 h (Fig. 3; Table 4; $P < 0.001$).

To search for endocrine-immune relationships, the means between 0800 and 2000 h values were calculated for immune variables and plasma ACTH in pituitary- or adrenal-dependent patients. A significant positive correlation was apparent in pituitary-dependent Cushing's patients between mean levels of spontaneous NK activity and mean plasma ACTH ($r = 0.64$, $P < 0.02$; Fig. 4A). In addition, a significant inverse correlation was obtained between mean cortisol-dependent inhibition of spontaneous activity (percentage values) and mean plasma ACTH ($r = -0.69$, $P < 0.02$; Fig. 4B).

No correlation was observed between mean levels of spontaneous cytotoxicity and urinary free cortisol in the pituitary-dependent group of patients (Fig. 5A), whereas it was apparent in the adrenal-dependent group ($r = -0.67$, $P < 0.02$; Fig. 5B). It should be noted that the patient who appears as outlier in Fig. 5B was one of the patients affected with adrenal-dependent Cushing's syndrome having the greatest serum cortisol concentration and spontaneous cytotoxicity values far below the means observed in this group (0800 h: 0.9 LU/10⁷ PBMC; 2000 h: 4 LU/10⁷ PBMC).

Discussion

Our data are the first to deal with NK cell activity in Cushing's syndrome as a function of different aetiologies and hence divergent patterns of ACTH and other POMC-derived peptides in the face of glucocorticoid excess. Recent work by Kronfol *et al.* (22) indicating a reduced NK cell activity in Cushing's patients, did not take into account the aetiology of the glucocorticoid excess.

As a general feature, at 0800 h our Cushing's patients displayed significantly lower levels of spontaneous NK cell activity than did controls, in agreement with reported data (22). It is established that glucocorticoids interfere profoundly with the function of many immunocytes (23, 24). As one might expect,

Figure 3 *In vitro* responsiveness to cortisol (10⁻⁶ mol/l) of PBMC preparations obtained at two opposing circadian stages (0800 and 2000 h) from healthy subjects (controls) and patients with Cushing's syndrome, either pituitary-dependent or adrenal-dependent. Data are expressed as percentage changes (mean $\pm$ S.E.) from spontaneous activity of PBMC preparations taken as 100. *** $P < 0.001$ compared with values obtained at 0800 h.

Figure 4 (A) Correlation between means of 0800 and 2000 h spontaneous NK cell activity and plasma ACTH in patients with pituitary-dependent Cushing's syndrome. Data are validated by Spearman's rank correlation test: $r=0.64$; $P<0.02$. (B) Correlation between means of 0800 and 2000 h cortisol-dependent inhibition of spontaneous NK cell activity and plasma ACTH in patients with pituitary-dependent Cushing's syndrome. Data are validated by Spearman's rank correlation test: $r=-0.69$; $P<0.02$.

chronic endogenous glucocorticoid excess also reduces natural cytotoxicity. It should be pointed out that, as found by other authors (22), a count of circulating NK effectors by surface phenotype analysis did not reveal any abnormality of the number of CD56⁺ cells. It is arguable that, *in vivo*, cortisol excess affects the biochemical machinery responsible for cytotoxicity (10), while sparing cell integrity and viability. Extensive studies have demonstrated that both the spontaneous activity and the cortisol-dependent effects on NK cytotoxicity are mediated, to a remarkable extent, by Ca²⁺-dependent pathways (10). Abnormalities of intracellular calcium traffic might be responsible for glucocorticoid-mediated immune alterations.

Figure 5 (A) Correlation between means of 0800 and 2000 h spontaneous NK cell activity and urinary free cortisol in patients with pituitary-dependent Cushing's syndrome. Data are validated by Spearman's rank correlation test: $r=-0.32$; ns, not significant. (B) Correlation between means of 0800 and 2000 h spontaneous NK cell activity and urinary free cortisol in patients with adrenal-dependent Cushing's syndrome. Data are validated by Spearman's rank correlation test: $r=-0.67$; $P<0.02$.

Levels of spontaneous activity were greater in pituitary-dependent Cushing's patients than in adrenal-dependent ones. This result is conceivably due, not only to lower serum cortisol in the first group of patients with respect to the latter, but also to greater plasma ACTH concentrations. POMC-derived peptides have been shown to modulate *in vitro* NK cell activity positively and, notably, to potentiate the effects of stimulatory cytokines (11, 12). In this regard, our data can be viewed as compatible with a positive immunomodulatory role for ACTH *in vivo* also, consistent with previous observations in patients with anorexia nervosa, in whom the magnitude of ACTH release after corticotrophin-releasing hormone challenge was correlated with incremental NK cytotoxicity after IL-2 (25).

The observation, in the present study, of a negative correlation between percentage cortisol-dependent inhibition and plasma ACTH in pituitary-dependent cases could be an additional piece of evidence for the ability of the pituitary peptide to effectively counterbalance *in vivo* glucocorticoid-induced immunosuppression, as it has also been documented *in vitro* (12). In adrenal-dependent patients, we observed a negative correlation between levels of spontaneous NK cell activity and urinary free cortisol. Although statistical significance could be affected by the presence of an outlier patient, our data are consistent with the view that the immunosuppressive properties of unopposed glucocorticoids exert greater influence (6, 12).

No definitive conclusions can be drawn from our two patients with ectopic ACTH production. In such patients, concentrations of circulating glucocorticoids are conceivably too great to be counterbalanced by POMC-derived peptides of neoplastic origin.

As for the spontaneous NK cell activity, rIFN- γ -dependent enhancement was lower in patients with Cushing's syndrome than in controls. Interrelationships between glucocorticoids and cytokines have been a focus of extensive research (26). In addition to decreasing production of cytokines, glucocorticoids interfere with their action by inhibiting cytokine-dependent phosphorylation of several intracellular proteins or affecting synthesis of soluble or cellular specific receptors (27–29). We can not exclude that, in our patients, hypercortisolism resulted in analogous inhibition of the IFN- γ system. It remains to be elucidated whether IFN- γ release, IFN- γ receptor integrity, or both, are impaired.

As far as *in vitro* responsiveness to cortisol is concerned, PBMC obtained from Cushing's patients were less sensitive to the steroid than were those from control subjects. A lower degree of *in vitro* effectiveness of the hormone in Cushing's patients could be due to reduced availability of specific receptors on mononuclear cells as a consequence of endogenous hypercortisolism. In this view, recent literature reports the decrease in corticosteroid sensitivity of peripheral immunocytes exposed to excess endogenous or exogenous glucocorticoids (29, 30). In such acute conditions serum cortisol levels are often comparable to those observed in Cushing's patients. Further studies are needed to determine the molecular mechanisms of glucocorticoid resistance in these circumstances.

Notwithstanding limitations due to our consideration of only two time points over the course of the 24-h day, our results also suggest that, in Cushing's patients, diurnal variations of spontaneous NK cell activity and *in vitro* responsiveness to physiological modifiers are lost. It is held that the HPA system is strongly organized according to a circadian programme and can be considered to be one important endogenous synchronizer of many bodily functions, including immune reactions among others (16, 31, 32). It is not

surprising, therefore, that, in the presence of profound abnormalities of the circadian pattern of HPA hormones, diurnal immunological variations are no longer apparent.

To conclude, our data demonstrate that, in patients with Cushing's syndrome, NK cell activity is impaired. The degree of NK cell impairment is apparently greater in patients with autonomous adrenal hypersecretion, in whom glucocorticoid effects are unopposed by ACTH. Overall, our results provide an additional piece of evidence for the role of HPA hormones in the modulation of natural cytotoxicity *in vivo*.

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