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Spontaneous and Cytokine-Induced Natural Killer Cytotoxicity in Patients With Cushing's Disease

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Abstract: Patients with endogenous hypercortisolism are prone to develop infectious diseases. In this study, we investigated the spontaneous and in vitro response to interleukin-2 (LAK) or interferon-α (IFN-α NK) natural killer cytotoxicity in 12 ambulatory female patients with Cushing's disease (CD). Twelve healthy age-matched women served as control subjects (C). Mean spontaneous NK activity in CD (1.82 $\pm$ 0.77 LU/10^6 PBL) was not different than C (2.38 $\pm$ 0.59 LU/ 10^6 PBL). Mean LAK activity in CD (4.27 $\pm$ 1.72 LU/10^6 PBL) was significantly lower than C (9.52 $\pm$ 1.19 LU/10^6 PBL; $P < 0.001$). Mean IFN-α NK cytotoxicity response in CD (3.33 $\pm$ 1.57 LU/10^6 PBL) was significantly reduced compared with C (8.41 $\pm$ 1.24 LU/10^6 PBL, $P < 0.001$). Spontaneous and stimulated NK cytotoxicity did not show correlation with plasma corticotropin, total urinary-free cortisol, or late-evening salivary cortisol. Although spontaneous NK cytotoxicity did not demonstrate differences from healthy women, this finding did not exclude an impairment of the NK response to immunomodulators like found in these high-risk patients.

Key Words: natural killer cytotoxicity, Cushing's disease, interleukin-2, interferon-alpha

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Learning Objectives:

- Compare spontaneous and cytokine-induced natural killer (NK) lymphocyte cytotoxicity in women with active Cushing's disease and matched control subjects.
- Describe the relationship, if any, between spontaneous and lymphokine-activated NK cytotoxicity and hormonal parameters of cortisol excess in the CD and control groups.
- Offer a credible explanation of the observed *in vitro* effects of excess glucocorticoid on spontaneous and cytokine-induced NK cytotoxicity.

Natural killer cells (NK) have been defined as effector cells of nonadaptive natural immunity playing an important role in host defense against pathogens. NK cells have been described as a third group of lymphocytes that differs from T and B cells in surface phenotype, target recognition and function and comprise 5.0% to 15% of circulating human lymphocytes.[1–3] NK cells exert their antimicrobial action through the secretion of cytokines and cytotoxic activity. It has been shown that NK cells have antiviral, antifungal, and antibacterial activity in vitro; associating a marked reduction in NK activity to recurrent viral or bacterial infections.[1,4]

NK cell function is largely modulated by growth factors and cytokines. NK cell lytic activity has been shown to be regulated in vitro and in vivo by activating signals such as interferons (IFN-α, IFN-β, and IFN-γ) and interleukin-2 (IL-2).[5]

It has been reported that glucocorticoids exert multiple effects on lymphocytes, which include apoptosis induction, repression of genes that encode lymphokines, and suppression of cytotoxic activity[6]. Glucocorticoids are able to lower human NK lytic activity[7,8]; we previously demonstrated that cortisol inhibited spontaneous and IL-2-activated NK cytotoxicity in vitro.[9] This effect could be mediated through its action on the corticosteroid receptor described in NK cells[10]; however, the molecular mechanisms of glucocorticoid-mediated suppression of NK cytotoxicity has not been completely understood.

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Patients with endogenous cortisol excess demonstrate immune dysregulation. They are susceptible to opportunistic pathogens, and systemic infections are among the principal causes of mortality in these patients.[11–14]Few reports focused on the study of NK cytotoxicity in patients with Cushing's syndrome. Kronfol and colleagues[15] and Masera and colleagues[16] found that spontaneous NK cytotoxicity was reduced in patients with Cushing's syndrome. However, the NK cytotoxic response to immunomodulators has not been extensively studied. We found only one report in which NK cytotoxicity was demonstrated to be reduced in response to interferon-gamma in patients with endogenous Cushing's syndrome.[16]

The aim of our study was to further investigate, in female patients with active Cushing's disease, the spontaneous NK cytotoxicity from peripheral blood lymphocytes and the ability of NK cells to be activated by IL-2 (LAK response) and IFN-α (IFN-α-NK) in vitro.

METHODS

Subjects

We studied 12 female patients (aged 18–42 years) with Cushing's disease (CD). The biochemical diagnosis was performed through the detection of high total excretion of urinary cortisol (F_{24}), elevated nocturnal salivary cortisol (SAF_{23}), and the absence of cortisol suppressibility after 1 mg of dexamethasone administered overnight.[14,17] An overnight 8-mg dexamethasone suppression test was performed in all cases, and cortisol levels fell to less than 50% of baseline values. Corticotropin (ACTH) levels ranged from 4.2 to 40.7 pmol/L (Table 1). All the patients had active disease and had not received any previous therapy. The duration of disease ranged from 2 to 5 years. Amenorrhea was present in all the cases with a duration range from 1 to 5 years. Patients were ambulatory, euthermic, and none developed severe infections. In 3 cases (patient nos. 4, 5, and 6), onychomycosis (*Candidiasis*) was present and patient no. 6 had associated submaxillary sinusitis.

The control group was comprised of 12 healthy female volunteers (aged 20–45 years), free of endocrine and psychiatric illnesses and with regular menses (Table 1). Each control subject was paired by age with a patient.

All subjects were informed of the purpose of the study and gave their consent to perform it.

Study Design

The day before the study, all the subjects collected 24-hour urine for cortisol measurement (F_{24}). In addition, whole saliva samples were collected for nocturnal salivary cortisol determination at 11:00 pm (SAF_{23}) as described by Raff et al.[17]

TABLE 1. Biochemical Parameters in Women With Cushing's Disease (n = 12)

Patient no.	Corticotropin (pmol/L)	Nocturnal salivary cortisol (SAF23) nmol/L	Total urinary cortisol (F24) nmol/L	Whole blood count ($\times 10^9$/L)
1	23.1	21.0	222.0	8.0
2	19.8	20.0	231.0	8.4
3	4.2	18.0	263.0	9.3
4	40.7	15.0	237.0	11.3
5	19.8	25.0	265.0	7.0
6	11.0	22.0	524.0	13.5
7	33.2	15.0	248.0	8.5
8	13.2	40.0	239.0	13.7
9	9.9	12.0	215.0	10.1
10	16.0	10.0	183.0	7.8
11	6.6	9.2	717.0	16.7
12	8.8	22.0	966.0	11.9
Mean ± SD	17.2 ± 11.0‡	19.1 ± 8.3‡	359 ± 246†	10.5 ± 2.9*
Range	4.2–40.7	9.2–40.0	183–966	7.0–16.7
Control subjects				
Mean ± SD	4.82 ± 1.82	2.43 ± 1.43	81 ± 45	7.2 ± 1.58
Range	2.20–7.7	0.8–5.2	36–164	4.8–10.0

*$P = 0.003$ compared with control subjects.
†$P < 0.001$ compared with control subjects.
‡$P < 0.0001$ compared with control subjects.
SD, standard deviation.

At 9.00 am, 25 mL of blood was drawn from patients and control subjects in sterile conditions. Blood samples were processed for hemogram, ACTH, and natural killer cytotoxicity. Control women were studied during the follicular phase of their menstrual cycle.

Endocrine Measurements

Total urinary cortisol and salivary cortisol were measured using a commercial solid-phase radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). The intra- and interassay coefficients of variations were 4% and 6%, respectively, and the sensitivity 0.2 μg/dL in the F_{24} assay.

Salivary cortisol was determined in the supernatant obtained after the centrifugation of the whole saliva sample as described by Cardoso et al.[18] Intraassay and interassay coefficient of variation were less than 6% and 13%, respectively. The minimal SAF concentration detected was 0.5 nmol/L.

Plasma ACTH was assessed by immunoradiometric assay using a commercial kit from Nichols Institute Diagnostics (CA). The intraassay and interassay variations were 3.2% and 7.8%, respectively, and the sensitivity was 1 pg/mL.

Hemogram

White blood cells (WBC) were measured automatically on EDTA-anticoagulated blood in a Coulter JT (FL).

Cell Preparation

Lymphocyte Separation

Peripheral blood samples from patients and controls (15 mL) were collected in sterile heparinized tubes. Blood was diluted 1-fold and layered over Ficoll-Hypaque. Density gradient centrifugation was performed at 400 x g for 30 minutes. Mononuclear cells were recovered from the interface of the gradient. After 3 washings, cells were suspended in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gen S.A., Argentina), glutamine (5 mg %), streptomycin (0.1 mg/mL), and sodium bicarbonate solution (0.2%), and incubated in plastic Petri dishes at 37°C for 2 hours in a humidified atmosphere containing 5% CO_2 in air. Nonadherent cells were collected, washed once with the culture medium (CM), and the macrophage content was determined by esterase staining. The percentage of monocytes was less than 2%. Total lymphocytes (PBL) were adjusted to a final concentration of 1 x 10^6/mL in CM. Cellular viability in all cases was ≥98%.

Treatment of Effector Cells

PBL were incubated in CM alone and either with 125 IU/mL of human recombinant interleukin-2 (Gibco BRL) or 1000 IU/mL of interferon-α_2 (Roche, Argentina). After 18 hours of incubation under the conditions previously described, PBL were washed twice and were used as effector cells in the cytotoxicity assay to evaluate NK activity, lymphokine-activated killer cell activity (LAK), and NK IFN-α response (IFN-NK).

TABLE 2. Spontaneous and Activated Natural Killer (NK) Cytotoxicity Response by Interleukin-2 (LAK) and Interferon-α (IFN-α-NK) in Patients With Cushing's Disease (CD) and Healthy Women

Subjects	NK (LU/10^6 PBL)	LAK (LU/10^6 PBL)	IFNα-NK (LU/10^6 PBL)
Patients			
1	3.20	6.76	6.26
2	1.66	5.40	4.08
3	2.17	5.82	3.28
4	1.16	2.95	1.59
5	0.90	1.80	1.60
6	1.00	3.25	2.22
7	3.00	6.25	5.00
8	2.31	5.00	5.31
9	1.70	2.20	2.12
10	2.00	4.20	2.93
11	0.90	2.30	1.80
12	1.80	5.30	3.72
Mean ± SD	1.82 ± 0.77	4.27 ± 1.72*†	3.33 ± 1.57*†
Range	0.90–3.20	1.80–6.76	1.59–6.26
Control subjects			
1	3.12	11.00	10.00
2	1.66	8.36	8.00
3	2.00	9.37	8.10
4	2.40	10.00	9.00
5	3.40	12.00	9.00
6	2.86	10.00	8.60
7	3.00	10.00	11.00
8	1.76	7.66	6.80
9	1.94	8.90	7.60
10	2.20	9.50	7.00
11	1.76	8.50	7.20
12	2.40	9.00	8.60
Mean ± SD	2.38 ± 0.59	9.52 ± 1.19‡	8.41 ± 1.24‡
Range	1.66–3.40	7.66–12.00	6.80–11.00

*P <0.001 compared with control subjects.
†P <0.01 compared with spontaneous NK activity in patients with CD.
‡P <0.001 compared with spontaneous NK activity in control subjects.

Cytotoxicity Assay

The K562 cell line[19] was the target cell in the ^{51}Cr release assay to examine the standard natural activity of the PBL. Target cells (T) were incubated for 1 hour at 37°C with 100 μCi of $^{51}CrO_4Na_2$ (New England Nuclear, Dupont, Argentina) and extensively washed before use. The cytotoxicity assay was performed in triplicate, in each subject, in

96-well microtiter plates (Corning, NY). Labeled target cells (1 x 10^4 cells, 0.05 mL) were incubated with 0.1 mL of PBL to obtain an effector:target ratio of 100:1, 50:1, 25:1, and 12.5:1. The plates were spun for 3 minutes at 500 g and incubated for 4 hours in a CO_2 incubator at 37°C. Then the plates were spun 10 minutes at 500 g, and 0.1 mL of the supernatant was taken from each well. Chromium release was detected using an auto gamma Cobra II (Packard). The percentage of killed target cells was calculated as follows:

$$\text{Specific lysis percent} = \frac{\text{Experimental cpm [E]} - \text{Control cpm [C]}}{\text{Maximum cpm [M]} - \text{Control cpm [C]}}$$

where E means cpm chromium release in the presence of effector cells, C means cpm spontaneously released by target cells incubated with medium, and M means cpm of 0.1 mL aliquot of resuspended target cells. In the 4-hour assay, the ratio of spontaneous to maximum ^{51}Cr release was lower than 10%. The cytotoxic capacity of effector cells was determined by linear regression of the percentage of ^{51}Cr release against the E/T ratio.[20] A lytic unit is defined as the number of peripheral blood lymphocytes required to produce 20% of specific cytotoxicity in our system.[9] The cytotoxic activity is expressed in lytic units per 10^6 PBL (LU/10^6 PBL).

To evaluate the interassay variation of NK activity, we determined in every assay, like Ogata et al.,[21] the NK cytotoxicity from PBL of one volunteer simultaneously with patients and control samples. In all the experiments, the volunteer NK activity fell in the range of 2.90 ± 0.30 LU /10^6 PBL.

Statistical Analysis

Statistical analysis was performed by Mann-Whitney *U* test and paired Student *t* test. Hormone parameters and NK activity were correlated using Spearman rank correlation test. Results were considered statistically significant whenever *P* was ≤0.05.

RESULTS

The hormonal and hematologic profile of patients with CD is shown in Table 1. Urinary F_{24} and SAF_{23} were, as expected, statistically higher than in control subjects. ACTH plasma levels were above 7.7 pmol/L in 10 of 12 patients with CD. The mean WBC in CD was significantly higher than in control subjects ($P < 0.05$), although only 50% of the patients had leukocytosis (patient nos. 4, 6, 8, 9, 11, and 12). No correlation was found between WBC and F_{24}, $SAF_{23,}$ or ACTH.

Natural Killer Activity

Individual data of spontaneous and stimulated NK activity in patients with CD and control subjects are shown in Table 2.

Eight of 12 patients with CD demonstrated spontaneous NK activity in the normal range (≥1.66 LU/10^6 PBL). However, patient nos. 4, 5, 6, and 11 showed a reduction in baseline NK activity (less than 1.16 LU/10^6 PBL). Mean baseline NK activity in patients with CD was not different from control subjects (1.82 ± 0.77 LU/10^6 PBL vs. 2.38 ± 0.59 LU/10^6 PBL; *P* = not significant). Spontaneous NK activity did not show significant correlation with ACTH, $F_{24,}$ or SAF_{23} in either patients with CD or control subjects.

Lymphokine-Activated Killer Cell Activity

The maximal LAK activity observed in a patient with CD (patient no. 1) was 6.76 LU/10^6 PBL (Table 2). This cytotoxic value was lower than the minimal LAK activity found in control subjects (7.66 LU/10^6 PBL). In addition, mean LAK activity in those with CD (4.27 ± 1.72 LU/10^6 PBL) was significantly reduced than control subjects (9.52 ± 1.19 LU/10^6 PBL; $P < 0.001$). The mean percentage change of NK activity induced by IL-2 was statistically lower in those with CD (141.0 ± 57.0%) than control subjects (321.0 ± 76.0%; $P < 0.001$) (Table 3).

No statistical correlation was found between ACTH, F_{24}, or SAF_{23} and LAK activity in either patients with CD or control subjects.

TABLE 3. Percentage of Change of Natural Killer (NK) Cytotoxicity in Response to Interleukin-2 (LAK) and Interferon-α (IFN-α-NK) in Women With Cushing's Disease and Control Subjects

	Control Subjects		Patients with Cushing's disease	
	Basal Cytotoxicity (LU/10^6 PBL)	Change from Basal NK Activity (%)	Basal Cytotoxicity (LU/10^6 PBL)	Change from Basal NK Activity (%)
Spontaneous (mean ± SD)	2.38 ± 0.59		1.82 ± 0.77	
LAK (mean ± SD)	9.52 ± 1.19	321.0 ± 76.0	4.27 ± 1.72	141.0 ± 57.0*
IFN-α-NK (mean ± SD)	8.41 ± 1.24	264.0 ± 58.0	3.33 ± 1.57	83.4 ± 39.0*

*$P < 0.001$ compared with control subjects.
SD, standard deviation.

Natural Killer Interferon-α Response

All patients with CD showed IFN-α-NK responses lower than 6.26 LU/10^6 PBL. This cytotoxic activity was below the minimal IFN-α response found in control subjects (6.80 LU/10^6 PBL). The mean IFN-α-NK response (3.33 $\pm$ 1.57 LU /10^6 PBL) was significantly reduced compared with control subjects (8.41 $\pm$ 1.24 LU /10^6 PBL; $P < 0.001$) (Table 2).

The mean percentage change in control subjects after IFN- α treatment (264.0 $\pm$ 58.0%) was significantly higher than that observed in patients with CD (83.4 $\pm$ 39.0%; $P < 0.001$) (Table 3).

No correlation was observed between mean levels of IFN-α-NK activity and hormonal parameters of cortisol excess (ACTH, F_{24}, and SAF_{23}) in either those with CD or healthy subjects.

DISCUSSION

In this study, we show that basal spontaneous NK cytotoxicity is not significantly different between women with CD and control women. By contrast, all patients with CD demonstrated statistically lower LAK and IFN-α-NK responses. We did not find any correlation between the hormonal hyperactivity of the pituitary–adrenal axis and the spontaneous and/or cytokine-stimulated NK cytotoxicity.

The unaltered spontaneous NK cytotoxicity found in our study could be the result of positive NK immunomodulation exerted by excessive ACTH secretion in patients with CD, counteracting the suppressive effect of glucocorticoids on spontaneous NK activity. The evidence supporting a role of hypothalamic–pituitary adrenal hormones in the modulation of NK activity in which the suppressive effects of glucocorticoids is counterbalanced by positive signals from CRH or proopiomelanocortin-derived peptides is increasing.[22–24]

We previously showed that patients given long-term glucocorticoid therapy at supraphysiological doses had blunted LAK and IFN-α-NK responses in vitro.[25] These results agree with our present data suggesting that the inhibitory effect of glucocorticoids could become overt only when IL-2 and IFN-α activate the NK cell system.

Masera and colleagues[16] differentiated spontaneous and activated IFN-γ NK responses in 12 patients of both sexes with CD. The authors did not find a correlation between spontaneous NK cytotoxicity and total urinary cortisol concentrations in accordance with our observation. By contrast, they found a positive correlation between spontaneous NK activity and ACTH levels that we did not find. They also showed that mean spontaneous cytotoxicity in patients with CD was lower than in healthy subjects. An interesting observation was that spontaneous NK activity was greater in ACTH-dependent than ACTH-independent patients with CD. This finding agrees with our data on spontaneous NK cytotoxicity in women with CD.

Glucocorticoids alter the host response to both common and unusual infectious agents through widespread effects on the immune and inflammatory responses.[26]High circulating levels of either endogenous or exogenous glucocorticoids impair the host response, predisposing the patients to infectious complications with opportunistic pathogens (*Candida, Cryptococcus*, *Aspergillus*, Nocardia, *Pneumocystis carinii*, Toxoplasma, Cryptosporidium, herpes viruses, *Mycobacterium tuberculosis*, *Mycobacterium avium-intracellulare*, Listeria monocytogenes).[11,12,13,27] Sarlis et al.[27] described patients with Cushing's syndrome in which sepsis was positively associated with the degree of hypercortisolism (F24 $\geq$2000 μg/day). The moderate cortisol excess (F24 $\leq$700.0 μg/day) described in our patients could explain the absence of systemic infections in our group.

In conclusion, we demonstrate that women with active Cushing's disease have lower LAK and IFN-α-NK cytotoxicity than healthy women. Unaltered spontaneous NK cytotoxicity does not seem to predict the reduced response of NK cells to IL-2 and IFN-α. These data reinforce the concept of disimmunity described in patients with endogenous hypercorticism.

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