Increased Expression of the Glucocorticoid Receptor β in Infants With RSV Bronchiolitis

WHAT’S KNOWN ON THIS SUBJECT: Most studies on corticoid treatment of respiratory syncytial virus (RSV) respiratory diseases have revealed no beneficial effect. The mechanism by which RSV respiratory-infected patients are insensitive to the antiinflammatory effect of corticosteroids is unknown.

WHAT THIS STUDY ADDS: This study helps to understand how a respiratory syncytial viral infection may alter the normal antiinflammatory response to cortisol and the insensitivity to glucocorticoid treatment. The increase expression of β glucocorticoid receptor could be a marker of disease severity.

abstract

OBJECTIVES: The majority of studies on glucocorticoid treatment in respiratory syncytial virus (RSV) bronchiolitis concluded that there are no beneficial effects. We hypothesized that RSV-infected patients may have an increased glucocorticoid receptor (GR) expression, the isoform that is unable to bind cortisol and exert an antiinflammatory action.

METHODS: By using real-time polymerase chain reaction, we studied the expression of α and β GR in the peripheral blood mononuclear cells obtained from 49 RSV-infected infants (<1 year of age) with severe (n = 29) and mild to moderate (n = 20) illness. In plasma, we analyzed the level of cortisol by radioimmunoassay and inflammatory cytokines interleukin (IL)-10, IL-6, tumor necrosis factor-α, IL-1β, IL-8, IL-12p70, IL-2, IL-4, IL-5, interferon-γ, and IL-17 by cytometric beads assay. Statistical analysis was performed by nonparametric analysis of variance.

RESULTS: We found a significant increase of β GR expression in patients with severe illness compared with those with mild disease (P < .001) and with a group of healthy controls (P < .01). The α:β GR ratio decreased significantly in infants with severe disease compared with those with mild illness (P < .01) and with normal controls (P < .001). The expression of β GR was positively correlated with the clinical score of severity (r = .54; P < .0001).

CONCLUSIONS: The decrease of the α:β GR ratio by an increase of β receptors expression is related to illness severity and may partly explain the insensitivity to corticoid treatment in RSV-infected infants. The increased expression of β GR could be a marker of disease severity. Pediatrics 2012;130:e804–e811

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KEY WORDS
RSV bronchiolitis, infants, glucocorticoid receptor, interleukin-6, interleukin-8

ABBREVATIONS
ALRI—acute lower respiratory infection
GR—glucocorticoid receptor
IFN-γ—interferon-γ
IL— interleukin
mRNA—messenger RNA
NPA—nasopharyngeal aspirate
PBMC—peripheral blood mononuclear cell
RSV—respiratory syncytial virus
TNF-α—tumor necrosis factor-α

Dr Diaz contributed to the design of the protocol, analysis of results, and wrote the article; Drs Pinto and Mamani contributed to the clinical study of hospitalized infants and the selection and control of patients; Dr Uasapud contributed to the clinical study of ambulatory patients, the control of patients after hospitalization, and the selection of healthy controls; Dr Bono contributed to the acquisition of data on cytokines by cytometric beads assay and interpretation of data; Dr Gaggetto contributed to the acquisition of data on virus and the analysis and interpretation of data; and Drs Guerrero and Goecke contributed to studying glucocorticoid receptors and the analysis and interpretation of data. All authors made a critical revision of the article and authorized it to be published.

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Respiratory syncytial virus (RSV) infection is the primary cause of morbidity and mortality by respiratory viruses in infants all over the world. The severity of infection varies from a mild upper infection to a severe lower tract infection with bronchial obstruction, bronchiolitis, and bronchopneumonia. In Chile, ~2% of infected infants require hospitalization due to severe illness. The immunopathogenesis of RSV infection is not well understood, and no effective treatment is available. A shift to a Th-2 response due to a diminished interferon (IFN)-γ production by peripheral blood mononuclear cells (PBMCs) during severe RSV infection has been described and associated with an increase in endogenous production of plasma cortisol. The levels of plasma cortisol had a positive correlation with the severity of the infection and an inverse correlation with interleukin (IL)-12 and IFN-γ production by PBMCs.

The biological action of glucocorticoids is mediated by the glucocorticoid receptor (GR) α, an intracellular protein that binds with high affinity to cortisol. It is composed of 777 amino acids and is expressed in all types of cells. β GR is composed of 742 amino acids produced by splicing of the last exon; it is located in the nucleus, is unable to bind cortisol, and fails to activate transcription. Viral infections induce proinflammatory cytokines (IL-1, IL-6, IL-8, and tumor necrosis factor-α (TNF-α)), which activate the hypothalamus-pituitary-adrenal axis, releasing cortisol that has a potent antiinflammatory action. However, the effect of glucocorticosteroid treatment in RSV respiratory infections in children remains controversial. Most of the studies of RSV steroid treatments have revealed no beneficial effects. The mechanism by which bronchiolitis RSV-infected patients are resistant to steroid treatment is unknown. Primary culture of human bronchial epithelial cells infected with RSV and parainfluenza virus induces production of macrophage inflammatory protein-1α (MIP-1α) and IL-8. The addition of hydrocortisone attenuated cytokine production by parainfluenza virus but had no effect on RSV-infected cells. A direct effect of RSV on the GR has been recently described in the A549 and BEAS-2B cell lines derived from lung epithelium. The effect was direct, reducing GR binding to DNA and consequently inhibiting gene activation. Another mechanism involved in corticoid steroid insensitivity is the imbalance in α/β isoforms of the GR observed in the PBMC and bronchoalveolar lavage cells from glucocorticoid insensitive asthma patients. None of these mechanisms has been studied in RSV-infected patients. GR gene expression can be modulated by proinflammatory cytokines, such as TNF-α and IL-1, which in turn increases the β GR expression level over the α GR isoform. This unbalanced ratio correlates with glucocorticoid resistance.

We postulated that an imbalance in the isoforms α/β of glucocorticoids may be related to illness severity in RSV bronchiolitis and may partly explain the resistance to steroid treatment.

METHODS

We selected infants with RSV infection of different severity with symptoms (cough, nasal discharge, and fever) starting in the previous 3 days. All had respiratory distress with bronchial obstruction demonstrated by wheezing and/or hyperinflation in chest radiograph and positive RSV antigen detected by indirect immunofluorescence assay in nasopharyngeal aspirates (NPAs) or real-time polymerase chain reaction. They were term born without a previous history of any other significant pathology before the RSV infection. The inclusion criteria included infection with other viruses than RSV and having received corticosteroids in any formulation concomitant with the disease. The control group was selected from healthy infants who required blood tests as a requisite for minor surgery; their parents were contacted at the outpatient surgery clinic of the hospital. They were evaluated during a non-RSV epidemic period. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Chile and Roberto del Río Hospital. The parents gave informed consent for their infants to participate in the study after the aims and scope of the project had been explained to them.

Viral and Blood Studies

The preliminary study of NPA samples obtained from healthy controls as well as patients included polymerase chain reaction for RSV and immunofluorescence assay detection for RSV, parainfluenza 1, 2, and 3, influenza A and B, adenovirus, and metapneumovirus to detect dual infection. When a virus was detected in controls or a virus other than RSV in patients, they were not included in the study. From every subject, a second NPA sample with a blood sample was obtained in the morning, 24 to 48 hours after the diagnosis of RSV acute lower respiratory infection (ALRI) was made. A second blood and NPA sample was taken 1 month later when they were in good health.

Classification of Clinical Severity

The clinical criterion applied to define severity of the ALRI at admission into the hospital and throughout hospitalization was a slightly modified scoring system validated in the Roberto del Rio Children’s Hospital. The clinical severity was scored 1 to 8 according to the behavior of the infection. RSV-infected patients with mild ALRI and no need of hospitalization scored 1; when patients were hospitalized, the need for supplemental...
oxygen was between 24 and 72 hours, and the fraction of inspired oxygen was 22% to 30%; the score varied from 2 to 5. Patients with length of stay and duration of symptoms of 4 days or more, oxygen requirements of 4 days or more, and with a fraction of inspired oxygen >31% were scored from 6 to 8.

Blood Collection
Heparinized blood (3–5 mL) was collected between 8 AM and 9 AM from all participating infants for total leukocytes and differential counts. One milliliter of plasma was separated and kept frozen at -20°C for cytokines and cortisol assays.

GR Assay in PBMC
Mononuclear cells were separated from heparinized blood by Ficoll-Hypaque to identify GRs, α and β GR isoforms in infants with bronchiolitis and control infants, and were measured as follows: the reverse transcription reaction was performed on 2 μg of total RNA as previously described.21 The content of α and β GR messenger RNA (mRNA) was determined by using human 18S transcript from each complementary DNA sample as an internal standard and normalizing the results.

Cytokine Assay
Cytokine plasma concentration was determined by using a CBA kit (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s protocol. We evaluated IL-10, IL-6, TNF-α, IL-1ß, IL-8, IL-12p70, IL-2, IL-4, IL-5, IFN-γ, and IL-17 concentrations by flow cytometry (FACScan II with DIVA software, BD Biosciences, San José, California).

Cortisol Assay
The concentration of plasma cortisol (ng/mL) was determined by radioimmunoassay by using Cortisol Radioimmunoassay Kits (Diagnostic System Laboratories, Inc, Webster, TX).

Statistical Analysis
Medians were analyzed by using non-parametric Kruskal-Wallis analysis of variance, followed by Dunn’s multiple comparison test to compare 3 groups, and the Mann-Whitney U test for pairs of groups, using the Graph Pad InStat program (Version 3.05 Created September 27, 2000, Registered to laboratory Universidad de Chile. GTA33483-833). Correlations were analyzed by Spearman’s rank correlation test. P < .05 was accepted as statistically significant.

RESULTS

Demographic and Clinical Data
We studied 29 infants (16 boys) with severe infection (score, 6–8), with a median age of 3 months (2–6 interquartile range), who were admitted to the Roberto del Rio Children’s Hospital in Santiago, Chile. None received mechanical ventilation or required antibiotic treatment. This group was compared with 20 infants who had a mild to moderate infection (14 boys) with a median age of 3.5 months (3–6 interquartile range) with no need of hospitalization (12 patients) or with a short stay in the hospital with supplemental oxygen for 3 days or less as described (8 patients).

GR in PBMC
We first evaluated GR expression in severe, mild to moderate RSV-infected patients and control infants. Figure 1 shows in logarithmic scale median and individual values of GRs α (Fig 1A), β (Fig 1B), the α:β ratio (Fig 1C), and the α:β ratio receptors from 12 patients during the acute phase of the RSV infection and 1 month later (Fig 1D).

Figure 1A shows that the median values of α GR were 1.320 × 10⁻², 0.431 × 10⁻², and 0.676 × 10⁻² in patients with severe illness, mild illness, and controls, respectively. No significant differences were found between these values. Figure 1B shows the median and individual values of the β isoform of the receptor. We found in 29 patients with severe RSV bronchiolitis an increase in mRNA of 0.0908 × 10⁻², significantly greater than the median of 0.00040 × 10⁻² obtained from 20 patients with mild disease (P < .001) and with the median of 0.00170 × 10⁻² observed in 25 healthy controls (P < .01). No significant differences were found between the group of patients with mild infection and the controls. Figure 1C shows a significantly lower median of the ratio of α/β glucocorticoid receptors in infants with severe disease 71 × 10⁻² compared with infants with mild illness, a median ratio of 1029 × 10⁻² (P < .01), and with the healthy controls median ratio of 566 × 10⁻² (P < .001). Finally, 12 of 29 patients who had a severe illness were accepted to be enrolled 1 month after the infection (Fig 1D). They had a significant increase (P < .01) in the α:β ratio from a median of 25 to 67.

Percentages and Absolute Values of Total Leukocytes, Lymphocytes, and Monocytes From Peripheral Blood in Patients With RSV and Controls

The differential cell count from peripheral blood revealed that the percentages of lymphocytes expressed as medians had significant differences from 48% in severely infected patients, 53% in patients with a mild to moderate infection, and 60% in controls (P < .001; P < .05, respectively, to controls), but when these percentages are expressed as absolute values, no significant differences were found. The median of percentages and absolute values of monocytes were not significantly different between the 3 groups (data not shown).
Next, we correlated the score severity of RSV-infected infants with α and β GR expression and with IL-6 and IL-8, both cytokines increased in plasma obtained from those infants. Figure 2A shows that the clinical severity of the severe and mild RSV-infected patients had a positive correlation with the expression of β receptors (r = .54; P < .0001) shown in Fig 2B. On the other hand, no correlation was found between clinical severity of the illness and α receptors or with the plasma levels of IL-8 (r = .39; data not shown).

Levels of Plasma Cortisol
As mentioned earlier, levels of plasma cortisol were positively correlated with illness severity. Figure 3 shows the median and individual values of plasma cortisol obtained from infants with RSV bronchiolitis and from healthy controls. Infants with severe illness had a median plasma cortisol level of 230.0 ng/mL significantly greater (P < .05) than the median levels of infants with mild disease of 123.0 ng/mL and significantly greater than controls who had a median of 49.0 ng/mL (P < .001 and .01, respectively).

Correlation of Plasma Cortisol With Plasma IL-6
In spite of the fact that GC down-regulates IL-6 production, we found a statistically significant (P < .0001) positive correlation (r = .55) of plasma levels of cortisol...
(ng/mL) obtained from RSV-infected infants with severe and mild to moderate illness and the production of plasma IL-6 (pg/mL) from the same patients (data not shown). We found no significant correlation with IL-8, the other cytokine increased in plasma, with the levels of plasma cortisol.

**Levels of Plasma Cytokines**

We found that only IL-6 and IL-8 increased in plasma obtained from RSV-infected infants in comparison with healthy controls (data not shown). None of the other cytokines quantified in plasma: IL-10, TNF-α, IL-1β, IL-12p70, IL-2, IL-4, IL-5, IFN-γ, and IL-17 had significant differences in the 3 groups.

**DISCUSSION**

We have shown in 49 infants aged <1 year, and for the first time in human beings, that during RSV respiratory infection there is an increase of mRNA of GC β receptors but not of the GC α receptors in mononuclear cells. The expression of β GR receptors in all subjects had great interindividual variability; however, a significant difference of the median value of the group was observed in patients with severe as compared with patients with mild infection and controls (Fig 1B). When we expressed the clinical severity in a continuous variable in Fig 2, all 12

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**FIGURE 2**

A correlation of clinical severity (score from mild [1] to severe [8]; x axis), with the expression of β GR isoform per $10^{-2}$ (A), expressed in logarithmic scale in the y axis and (B) plasma IL-6 (pg/mL) expressed in normal scale in the y axis. A, In 49 patients infected with RSV with different severities, we show a significantly ($P < .0001$) positive correlation ($r = .54$) of the score of clinical severity with the β GR per $10^{-2}$. Only 2 patients with mild infection (score 2) had a high mRNA expression of β GR, and 2 patients with severe infection (score 8) had a low mRNA expression of β GR. B, Clinical severity also reveals a statistically significant ($P < .0001$) positive correlation ($r = .52$) with the levels of plasma IL-6.
The level of plasma cortisol (ng/mL) shown as individual and median values in 29 severe, 20 mild to moderate RSV-infected patients, and in 25 healthy controls. The level of plasma cortisol obtained from patients with severe RSV infection was significantly greater than those of mild to moderate infection ($P < .05$) and healthy controls ($P < .001$).

![Figure 3](image-url)

**Figure 3**

The level of plasma cortisol (ng/mL) shown as individual and median values in 29 severe, 20 mild to moderate RSV-infected patients, and in 25 healthy controls. The level of plasma cortisol obtained from patients with severe RSV infection was significantly greater than those of mild to moderate infection ($P < .05$) and healthy controls ($P < .001$).

patients with mild disease who did not need to be hospitalized (score 1) had a lower level of $\beta$ GR expression as it is seen in Fig 2A. Comparing the $\beta$ GR expression of these 12 patients with very mild infection with the 8 patients who had a mild to moderate disease (scores 2–5) but were hospitalized, we found significant differences between them, with a median level of $0.000213 \times 10^{-2}$ in nonhospitalized patients versus $0.0037 \times 10^{-2}$ in the 8 hospitalized patients ($P < .0003$; data not shown). The increased expression of $\beta$ GR receptors and not in $\alpha$ GR receptors will result in a lower ratio of $\alpha/\beta$ receptors (Fig 1C). In fact, most patients with severe illness had a ratio of $\alpha/\beta$ GR lower than the median ratio found in mild patients and healthy controls. Even more, in the group of mild to moderate disease, the $\alpha/\beta$ GR ratio had the same behavior as the $\beta$ GR, with a higher ratio of 243 in the 12 nonhospitalized infants versus 53 in the 8 mild to moderate hospitalized patients ($P < .001$). These results indicate that the increased expression of $\beta$ GR is present in infected patients with more severe disease. Because the blood sample to measure mRNA of GR was taken at the beginning of the disease and we found a correlation of the $\beta$ GR with the evolution of illness (Fig 2A), we suggest that an increase in $\beta$ GR could help to predict which patients will have a more severe illness. We do not know whether during the course of the disease the amount of GR mRNA changes, but in the 12 patients evaluated during recovery of the illness, the $\alpha/\beta$ ratio of GR tends to increase (Fig 1D).

We do not know the cause of the increase in the $\beta$ GR expression of patients with severe RSV bronchiolitis. It is important to consider that GR were measured in mononuclear cells, and although we analyzed a well-established amount of mRNA, it was obtained from mononuclear cells with less percentages of lymphocytes in infected patients, and these cells differ from monocytes in their amount of expression of $\beta$ GR. However, we did not find a significant difference in the total amount of lymphocytes and monocytes between groups. The physiologic increase of glucocorticoids after stress has been described as a normal antiinflammatory response related to the up-regulation of $\alpha$ GR. In this study, we confirmed our previous findings of an increased level of plasma cortisol in infants with RSV infection (Fig 3). On the other hand, the increased expression of $\beta$ GR receptors, which has been shown to have a dominant negative effect, with no increase in $\alpha$ GR receptors, will determine a lower ratio of $\alpha/\beta$ receptors (Fig 1C), which has been associated with an insensitivity response to glucocorticosteroids. In fact, most patients with severe illness had a ratio of $\alpha/\beta$ GR lower than the median ratio found in mild patients and healthy controls. None of these patients was on steroids; therefore, we cannot confirm that they are insensitive to glucocorticoids treatment, but we could speculate that the more severe patients would be more resistant to treatment with steroids. It is possible that the increase in GC $\beta$ receptor will characterize a subgroup of patients with more severe disease.

Steroid insensitivity has been observed in other diseases such as some steroid resistant bronchial asthma. There are several mechanisms by which cells from steroid-resistant asthmatics have an impairment in the GR function and 1 of these is an increased level of $\beta$ GR. Bronchiolitis, as well as bronchial asthma, is a highly heterogenous disease; an increased level of $\beta$ GR expression in both cases may characterize a subgroup of patients who will not respond to steroid treatment.

A subtle balance between the inflammatory response to avoid viral replication and antiinflammatory action to avoid cell damage should be present, but it is not what we observed in RSV-infected patients. RSV is known to induce proinflammatory cytokines IL-6 and IL-8. In our study, we found a significant increase of IL-6 and IL-8 in plasma obtained from RSV-infected infants (data not shown in Figs) and in...
the NPA.\textsuperscript{27} The level of plasma IL-6 was positively correlated with the clinical severity of the patients ($r = .52; P < .0001$; Fig 2B) and with the level of plasma cortisol ($r = .55; P < .0001$), which reveals that the increase in endogenous plasma cortisol level does not induce a down-regulation of IL-6 or IL-8 in patients with severe RSV bronchiolitis. Interindividual variability in the response to inhaled steroids has been observed in asthmatic patients. The wide variability and the poor response to steroids treatment were associated with polymorphisms of the glucocorticoid-induced transcript 1 gene as recently described.\textsuperscript{28} One may speculate that the up-regulation of the GR response to RSV infection in some infected children may be due to polymorphisms of the GR receptors; however, in our study, the increased expression of the GR was inducible during the infection and tends to be reversible during convalescence, so it is unlikely that the effect would be due to a polymorphism.

**CONCLUSIONS**

We have demonstrated, for the first time, an increased expression of the GR isoform in patients with RSV bronchiolitis, which correlates with the severity of the disease. The increased expression of GR is present at the beginning of the disease and is greater in those patients who will have a more severe evolution. This finding could be an important clinical marker to identify this subgroup of patients with the worst course of the disease with a more prolonged and severe inflammatory response. It also may partly explain the insensitivity to glucocorticoid treatment and the controversial studies on glucocorticoid treatment on acute viral bronchiolitis. Moreover, it would suggest that glucocorticosteroid treatment in severe RSV bronchiolitis will not be effective and should not be given as a first choice.

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