A BRIEF COMMUNICATION

Upregulation of Interferon-Induced Genes in Infants with Virus-Associated Acute Bronchiolitis

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To determine whether there is an airway IFN response in infants with acute bronchiolitis and to establish whether the rate of such a response is related to the severity of illness, the expression of some IFN-induced genes was measured in nasopharyngeal washes from 39 infants with acute bronchiolitis. The results indicate that in infants with a virus-associated acute bronchiolitis there is a strong activation of IFN system and that the severity of illness is inversely related to the level of expression of IFN-induced genes. This suggests that the IFN response plays an important role in determining virus–associated respiratory disease in early life. Exp Biol Med 232:1355–1359, 2007

Key words: bronchiolitis; interferon; RSV; rhinovirus; PKR; 2–5 OAS; P56

Introduction

Acute bronchiolitis is a major cause of lower respiratory tract infection in infancy, and it is often caused by respiratory syncytial virus (RSV). Although knowledge of the immunopathogenesis of bronchiolitis caused by RSV has improved over previous decades, little is known about the association between viro/immunologic markers and disease severity. To this regard, interferon (IFN), which is produced by virtually all nucleated body cells when they become infected by viruses, appears to play a major role in inhibiting certain respiratory viral infections and in the recovery from related illnesses (1, 2). Published reports on the roles of IFN in respiratory illness are often conflicting and, specifically, there is very limited information about the activation of airway IFN response during acute bronchiolitis (2–4). Clinical studies have indicated that most children infected with RSV do not display detectable IFN levels in their sera and/or nasal secretions (3). On the contrary, other studies have shown that RSV strongly induces IFN or IFN-induced genes, such as IFI27, in airway epithelial cells and leukocytes (4). One of the issues that has not been addressed is whether the mounting of an appropriate airway IFN response is impaired in infants with RSV-bronchiolitis.

Changes in IFN-induced genes have been only marginally studied in infants with acute bronchiolitis (2–4) and have been never addressed in the respiratory tract.

In order to gain new insights into these issues, we evaluated the activation of three IFN-induced genes—2′-5′ oligoadenilate synthetase (2–5OAS), dsRNA-activated protein kinase R (PKR), and P56—in cells from nasopharyngeal washes from infants with a clinical diagnosis of acute bronchiolitis and correlated their expression levels with clinical severity.

This work was supported by a grant to G.A. from Sapienza University of Rome, Italy.

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Received May 8, 2007.
Accepted July 9, 2007.

DOI: 10.3181/0705-BC-124
1535-3702/07/23210-1355$15.00

Table 1. Severity Scores

	Severity score		
	0	1	2
Respiratory rate (breaths/min)	<45	45–60	>60
SpO_2 (%)	>95	95–90	<90
Retractions or nasal flaring	None	Retractions	Retractions + nasal flare
Respiratory sounds	None	Rales/wheezing localized	Rales + wheezing diffuse
Feeding	Normal	Reduce	Endovenous

Patients and Methods

A total of 39 infants (median age [range]: 60 days [10–270 days]; median weight [range]: 4.48 kg [2.45–10.53 kg]; male to female sex: 25:14; Caucasian: 85%) with a clinical diagnosis of acute bronchiolitis who were admitted from November 2005 to March 2006 to the Pediatric Department of Policlinico Umberto I Hospital were enrolled in this study. This study was approved by the Ethics Committees and/or Institutional Review Boards of the participating institution, and informed consent was obtained from the children's parents.

Disease severity and clinical evolution were evaluated by measuring the clinical score index as described by Wainwright *et al*. (5) in accordance with the respiratory rate, arterial oxygen saturation at room air, presence of retractions, radiologic findings, and feeding capacity (Table 1). Each infant was evaluated at the time of hospital admission and after 3 days.

Nasopharyngeal washes (NPWs) were collected in the first 48 hrs of admission to the hospital, and an aliquot was tested for virus detection as previously described (6). Briefly, a panel of polymerase chain reaction (PCR) or nested PCR assays, some of which were in a multiplex format, were used for detection of 14 respiratory viruses, including RSV, influenza A and B, coronavirus OC43, 229 E, NL63, HKU1, metapneumovirus, adenovirus, rhinovirus, and parainfluenza (1–4). The remaining NPWs were centrifuged at about 600 *g* for 10 mins, and the cell pellet was resuspended in 1 ml phenol and guanidine isothiocyanate reagent (Trizol; Gibco BRL, Grand Island, NY) and frozen at –80°C for subsequent IFN-inducible gene expression analysis. Specifically, the mRNA copy content of these genes was measured by a real-time 5′ exonuclease reverse transcription–PCR assay using the ABI 7700 sequence detector (Applied Biosystems, Monza, Italy). Total cellular RNA was extracted from the cells using Trizol reagent following the manufacturer's instructions, and it was retrotranscribed as previously described (6). Next, the primer pair and probes 2–5OAS (7), P56 (7), and PKR (forward primer, 5′-TGCTAC-TACGTGTGAGTCCCAAA-3′; reverse primer, 5′-TGATGTATCTGCTGAGAAGTCACCT-3′; probe, 5′-CAACTCTTTAGTGACCAGCACACTCGCTTCT-3′) were added to the universal PCR master mix (Applied Biosystems) at 300 and 100 n*M*, respectively, in a final volume of 50 µl. Co-amplification of the beta-glucuronidase gene (Assay-On-Demand, Hs99999908_m1; Applied Biosystems) was used to normalize the amount of total RNA present using the threshold cycle relative quantification according to the supplier's guidelines.

All results are expressed as median (range). Differences between infants with or without viral infections and RSV or rhinovirus infections, in terms of the level of IFN-induced genes in cells from NPW, were compared using the Mann-Whitney or the Kruskal-Wallis test, respectively. Spearman's rho coefficient was calculated in order to assess the correlation between the level of IFN-induced genes in cells from NPWs and the clinical score index. Mann-Whitney test was used for comparison of clinical score index between infants with RSV or rhinovirus infections. Pearson's *r* coefficient was calculated to assess the correlation between levels of different IFN-induced genes. The significance was fixed at the 5% level. Analysis was performed with SPSS v.13.0 for Windows (SPSS, Chicago, IL).

Results

A total of 39 specimens from children with acute bronchiolitis were examined for the presence of respiratory viruses. Respiratory virus infection was detected in 87% of the subjects. Specifically, the most frequently detected virus was RSV (68%), followed by rhinovirus (29%) and coronavirus OC43 (3%). A co-infection with two viruses, specifically RSV and influenza A, was recorded in only one patient.

Furthermore, the infants were divided into two groups on the basis of the absence (uninfected) or the presence of viruses in their NPWs, and the level of expression of IFN-induced gene in cells from NPWs was examined in both groups. The results indicate that the level of expression of all examined IFN-induced genes is highly variable in infants with acute bronchiolitis (coefficient of variation >100%) both in the presence and absence of viruses. Nevertheless, in the children with viral infections compared with uninfected infants, elevated levels of 2–5OAS (median [range]: 57.90 [1.21–1217.75] vs. 7.16 [1.52–22.94]); PKR (median [range]: 21.07 [1.35–198.09] vs. 3.63 [1.00–18.90]); and P56 (median [range]: 180.52 [1.00–2574.36] vs. 8.11 [1.00–30.91]) were found ($P < 0.05$). A direct positive correlation was found between 2–5OAS and all the other IFN-related genes (2–5OAS vs. PKR $r = 0.86$; 2–5OAS vs. P56 $r = 0.82$; $P < 0.05$), suggesting that these viral infections induce a coordinated activation of the IFN system. The

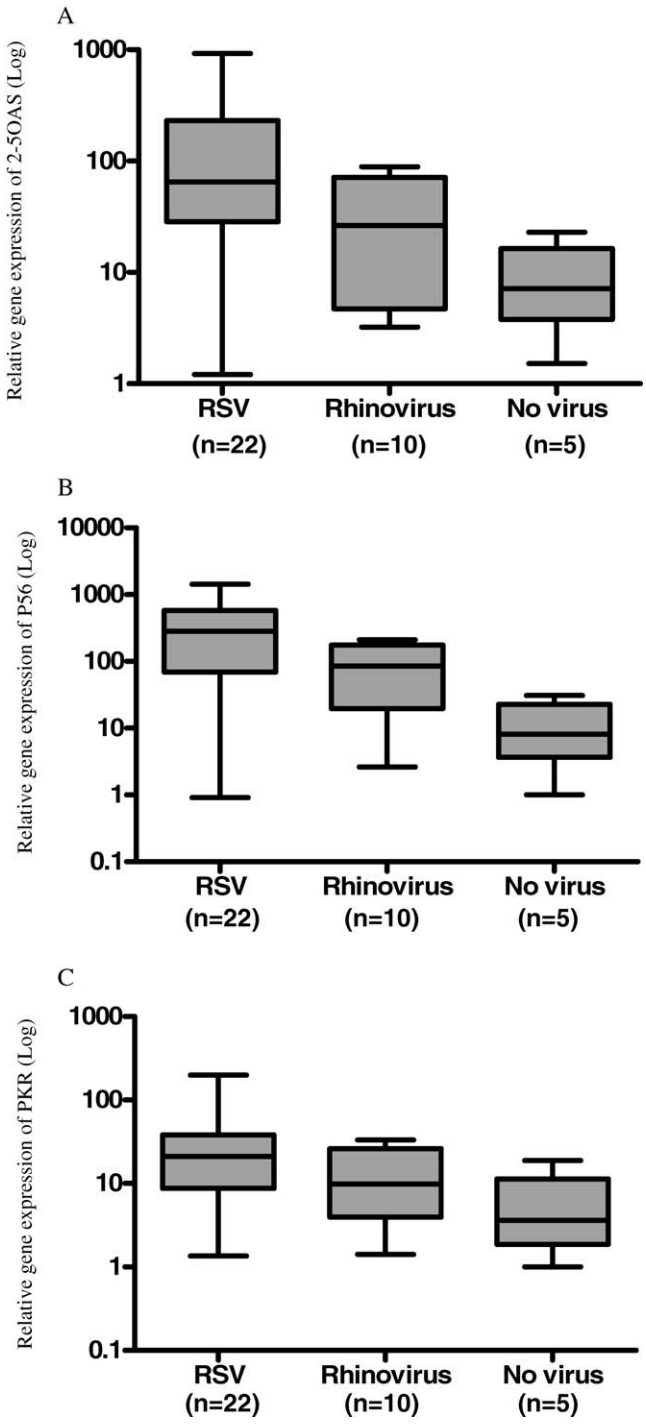

Figure 1. Relative gene expression of (A) 2′-5′ oligoadenilate synthetase (2–5OAS), (B) P56, and (C) dsRNA-activated protein kinase R (PKR) in infants with acute bronchiolitis in the presence (respiratory syncytial virus [RSV, $n = 22$] or rhinovirus [$n = 10$]) or absence of virus infection (no virus, $n = 5$). $P < 0.05$ by Kruskal-Wallis test.

above results were further analyzed in relation to the type of virus causing the infection. When patients were divided into three groups on the basis of the absence (uninfected) or the presence of RSV or rhinovirus infection, there was a significant difference ($P < 0.05$) between the groups. Patients with RSV showed higher expression of IFN-inducible genes compared with patients with rhinovirus or uninfected (Fig. 1). The differences are more evident when 2–5OAS and P56 are considered and are less pronounced in the case of PKR.

In addition to assessing whether expression levels of IFN-inducible genes are related to disease severity, we compared the gene expression of 2–5OAS, P56, and PKR with varying levels of disease severity, as determined by measuring the clinical score index. The results indicated that there was a weak but significant inverse correlation between the level of all three IFN-induced genes and the clinical score index (2–5OAS $r = –0.26$; P56 $r = –0.29$; PKR $r = –0.21$) recorded at the time of hospital admission (Fig. 2) in the infants with a virus-associated acute bronchiolitis.

The levels of IFN-induced genes in cells from NPWs were also analyzed with respect to the RSV or rhinovirus infection. A significant inverse correlation was observed between the clinical score index recorded at the time of hospital admission and the levels of IFN-induced genes (2–5OAS $r = –0.66$; PKR $r = –0.52$; $P < 0.05$) except for P56-mRNA ($r = –0.431$; $P = 0.07$) only in infants with RSV-associated bronchiolitis.

On the contrary, there was no significant correlation between the level of IFN-induced genes and the clinical severity clinical score index obtained in the analysis that was performed at the third day from the onset of symptoms (data not shown).

In addition, a comparison of clinical scores between RSV-infected children and rhinovirus-infected children revealed that there was greater severity of illness in RSV-positive children, although not at statistically significant levels (median: 4, range: 0–10; versus median: 3, range: 2–5).

Discussion

The present study confirms that viral infections are common in children with acute bronchiolitis. Our results also confirm that the frequency of RSV detection is high in this disease; the same data, however, point out that other respiratory viruses, mainly rhinovirus, may contribute to the pathogenesis of bronchiolitis as well. To our knowledge this is the first study that demonstrates a strong gene activation of the IFN system in the cells from NPWs derived from children with viral bronchiolitis. It is tempting to speculate that the accumulation of viral components such as dsRNA in epithelial cells and macrophages in the respiratory tract during viral replication and the potential for RSV or rhinovirus to cause productive infection in airway epithelial cells could be the main mechanisms to be taken into account to explain the strong gene activation of IFN response observed in infants with viral bronchiolitis (8). In agreement, it has been recently reported that both RSV and rhinovirus induce Toll-like receptor 3 protein, leading to increased dsRNA responsiveness in airway epithelial cells (9, 10). We cannot exclude, however, that the activation of

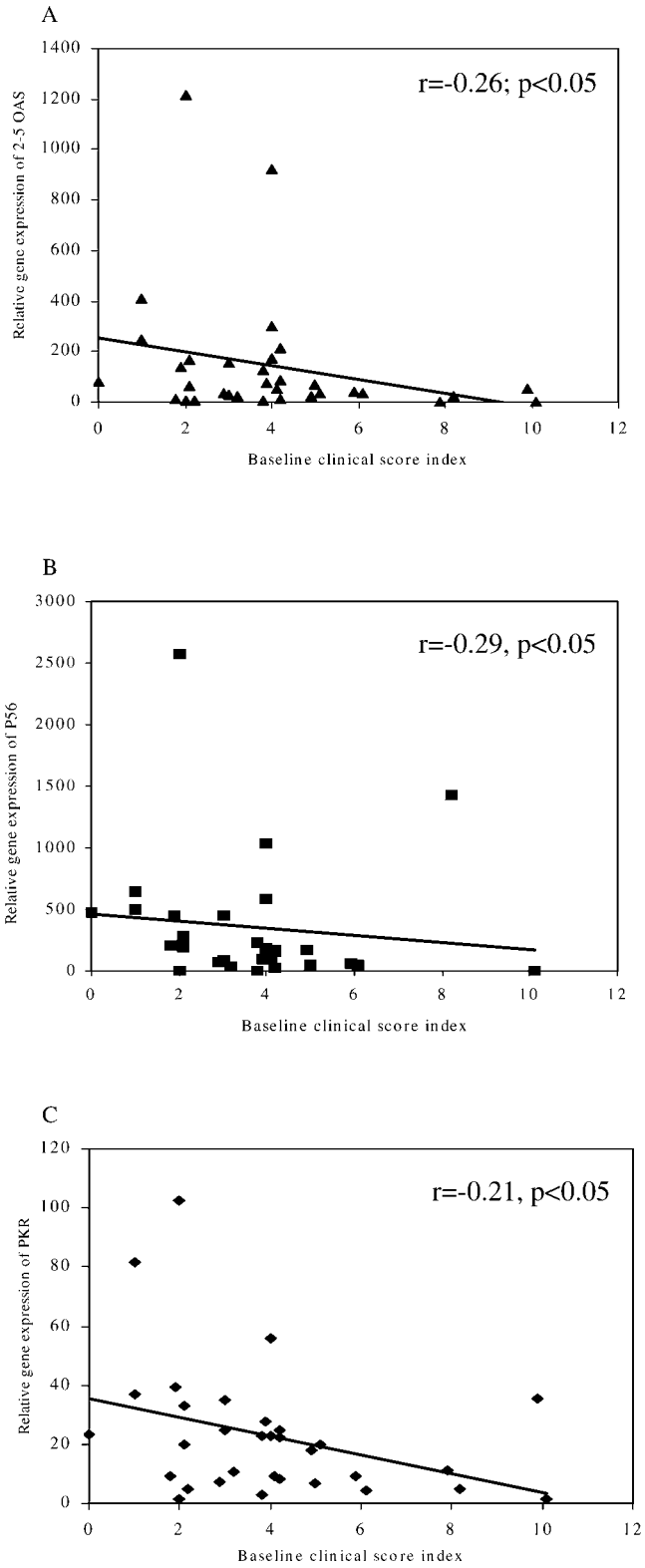

Figure 2. Relationship between the relative gene expression of (A) 2′-5′ oligoadenilate synthetase (2–5OAS), (B) P56, and (C) dsRNA-activated protein kinase R (PKR) in infants with virus-associated acute bronchiolitis and clinical index score. $P < 0.05$ by Spearman's rho test.

airway IFN response observed in our population could be the consequence of local production of cytokines and other mediators by epithelial cells and macrophages in the respiratory tract during viral bronchiolitis. Indeed, recent studies have suggested the involvement of IL-1, IL-8, IL-10, and TNF-alpha in regulation of antiviral activities by IFNs (11). Other reports have shown that the expression of many cytokines, including Rantes, MCP-1, MIP1-alpha, MIP1-beta, IL-6, IL-8, IL-10, and IL-16, were significantly increased in infants with bronchiolitis caused by RSV or rhinovirus compared with healthy controls (1, 12, 13). Therefore, our data could also suggest that viral infection elicits cytokine and chemokine production in the respiratory tract, which in turn leads to an increase in the synthesis of IFN, which would be critical for limiting and clearing respiratory viral infections.

Further studies are required to evaluate whether IFN responses reflect cytokine responses in the respiratory tract during viral bronchiolitis or are a direct consequence of viral replication.

Importantly, the results of this study also demonstrate that there was a trend toward greater expression of IFN-induced genes in RSV infections compared with rhinovirus infections. The latter would be in accordance with previous reports in which it has been shown that RSV is a potent inducer of IFN (4); the same data, however, are not consistent with other papers in which the production of IFN has been shown to be significantly reduced during acute bronchiolitis caused by RSV (3). This is in accordance with the finding that RSV possesses a mechanism to counteract type I IFN response. Specifically, recent studies have shown an important role for the RSV surface proteins in antagonizing the IFN-mediated antiviral response (14). As far as rhinovirus infection is concerned, it has been published that infection of rhinovirus results in the attenuation of the type I IFN response by disrupting activation of IFN regulatory factor 3, and thus inhibition of the early induction of IFN (15). This finding could be consistent with our results regarding the presence of higher alert status of the IFN system in infants with RSV than those with rhinovirus infections. The above apparent discrepancies along the data on the infant with OC43 infection who displayed a stronger expression of P56 and 2–5OAS compared with infants with RSV or rhinovirus infection (data not shown) make more evident the complexity of the analyzed phenomenon and the difficulty in the interpretation of the data. A better understanding of viral strategies for affecting IFN-dependent responses may allow for the mechanism underlying virus-induced airway disease.

The paper also indicates that the expression of IFN-induced genes in infants with viral bronchiolitis is inversely associated with the severity of disease. In agreement, it has been recently reported that deficient IFN-lambda production was highly correlated with severity of rhinovirus-induced asthma exacerbation (16). Moreover, Johnson and co-authors suggested that IFNs have an important role in

determining the nature and severity of RSV disease (2). The limited number of patients in our study, however, does not allow us to draw definitive conclusions about the role of P56, 2–5OAS, and PKR proteins in association with host response in bronchiolitis of moderate severity caused by virus.

Our results could suggest a scenario in which the antiviral IFN-inducible proteins are elicited early in response to respiratory virus replication during acute bronchiolitis. In this situation, elevated endogenous levels of the IFN-induced proteins were related to a moderate severity of bronchiolitis and *vice versa,* suggesting that the activation of IFN system plays an important role in determining virus-associated disease outcome.

The potential limitations of our study include the fact that we did not compare the level of expression of mRNAs encoding IFN-induced genes with the level of expression of other early activation genes not directly controlled by IFN in the cells from NPWs. This analysis could allow us to verify whether the correlation between the severity of bronchiolitis and the level of gene expression is specific for IFN-induced products or concerns other activation pathways. Unfortunately, the amount of sample was just enough to perform all the experiments shown in the present study, and the above issues could not be addressed. It would also be interesting to investigate whether there is a positive correlation between the level of expression of IFN-induced genes and the IFN level in NPWs or in the NPW-derived cells. Further studies specifically aimed to these important issues are needed.

Moreover, we did not assess whether the stronger expression of IFN-induced genes in infants with a moderate clinical course of virus-associated bronchiolitis was due to a better clinical response or to a low viral titer in their respiratory specimens. This is a very important issue. Indeed, recently it has been reported that there is a significant correlation between RSV viral load and the levels of proinflammatory cytokines in the nasal washes of children suffering from acute bronchiolitis (17). Moreover, a direct association has been observed between RSV viral load and the severity of bronchiolitis (18). Unfortunately, the limited amount of the samples did not allow us to address this important issue in the present study.

In conclusion, this study provides new insights into the activation of an IFN system in infants with acute bronchiolitis caused by virus and highlights the importance of studying these cytokine regulatory pathways *in vivo*. Larger longitudinal studies are required to gain a better understanding of the activation of an IFN system during acute bronchiolitis in early life.

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