Early high expression of IP-10 in F344 rats resistant to Sendai virus-induced airway injury

Xuezhong Cai and William L. Castleman

Department of Pathobiology, University of Florida, Gainesville, Florida 32610

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Cai, Xuezhong, and William L. Castleman. Early high expression of IP-10 in F344 rats resistant to Sendai virus-induced airway injury. Am J Physiol Lung Cell Mol Physiol 285: L1263–L1269, 2003; 10.1152/ajplung.00274.2002.—Weanling F344 and BN rats differ markedly in their susceptibility to Sendai virus-induced airway injury. Early gene expression that controls their differences in susceptibility remains poorly understood. In this study we combined suppressive subtractive hybridization and cDNA library array hybridization to identify genes differentially expressed in virus-susceptible BN and virus-resistant F344 rats during the first 3 days after inoculation. Differential expression of selected clones was further verified by quantitative RT-PCR. Seven virus-induced gene segments were identified. Of them, interferon-γ-inducible protein 10 (IP-10), Mx1, and guanylate-binding protein-2 mRNA abundance in infected F344 rats was 201.5, 188.2, and 281.7% higher, respectively, than that of infected BN rats at 2 days after inoculation. In situ hybridization indicated that virus-induced IP-10 was expressed mainly in airway epithelial cells of F344 rats. Sendai virus infection can directly induce IP-10 expression in rat tracheal epithelial cells in vitro. IP-10 early high expression might contribute to the resistance to virus-induced airway disease in F344 rats by promoting Th1 responses and increasing antiviral activity.

Respiratory viral infection during early life has been identified as an important risk factor in the development of asthma (10, 25). Asthma is characterized by variable and episodic airflow obstruction, airway inflammation, and bronchial hyperresponsiveness (10, 25, 36). There is strong evidence that asthma is controlled by multiple genes. However, the specific genes and their functions have not been completely characterized (36). Parainfluenza type 1 (Sendai) virus infection in inbred rat strains has been developed as an experimental model of asthma induced by viral infection during early life (5, 6, 15, 19, 26, 27, 32, 33). Virus infection during early life in susceptible BN rats results in acute bronchiolitis and airway injury that is followed by persistent airway inflammation and airway remodeling. Virus-resistant F344 rats develop acute airway inflammation following infection that resolves rapidly and is not accompanied by airway remodeling or airway dysfunction (32). BN rats develop chronic airway dysfunction in association with airway remodeling that is characterized by increased airway resistance and hyperresponsiveness to methacholine (15).

Comparative viral pathogenesis studies between BN and F344 rats have demonstrated that F344 rats have slightly more rapid clearance of Sendai virus from lungs than BN rats (27). Virus-susceptible BN rats develop persistent lymphocytic infiltrates in bronchioles following infection and have Th2-dominated cytokine responses that are associated with low interferon-γ (IFN-γ) responses (5, 27, 33). The greater susceptibility of BN rats to developing bronchiolar fibrosis and other remodeling changes that are associated with airway dysfunction is related to higher expression of tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) genes in bronchioles during chronic inflammation (32, 33). We hypothesize that other genes that are differentially expressed in the first 1–3 days after infection are critical in controlling resistance and/or susceptibility to virus-induced airway injury and development of subsequent asthma-like disease.

In this study, we used suppressive subtractive hybridization (SSH) (8) and cDNA library array hybridization to identify mRNA transcripts that were differentially expressed between BN and F344 rats from 1 to 3 days after Sendai virus inoculation. Quantitative RT-PCR assay and Northern blot analysis were performed to confirm the differential expression of target genes. In situ hybridization was used to localize expression of target genes in the lung.

Materials and Methods

Experimental strategy to identify susceptibility and resistance genes. This study focused on genes differentially expressed from 1 to 3 days after viral inoculation. Subtracted cDNA libraries were constructed for F344 and BN rats at 1, 2, and 3 days after virus inoculation in BN and F344 rats. Control BN and F344 cDNAs were used from unoinoculated rats at day 0 in the study. The cDNA analysis in this study can be simplified as the following steps:

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1) infected BN rat (BI) cDNAs – control BN rat (BC) cDNAs = virus-induced genes in BN rats + Sendai viral cDNAs [A]
2) infected F344 rat (FI) cDNAs – control F344 rat (FC) cDNAs = virus-induced genes in F344 rats + Sendai viral cDNAs [B]
3) A – B = virus-induced, differentially expressed susceptibility genes in BN rats
4) B – A = virus-induced, differentially expressed resistance genes in F344 rats.

Steps 1 and 2 were accomplished by SSH. Steps 3 and 4 were accomplished with cDNA library array hybridization to identify potential susceptibility and resistance genes.

**Rats and virus infection.** Male weanling BN/RijHsd and F344/NHsd rats (22 days old) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Eight rats from each infection group were inoculated at 25 days of age via aerosol exposure in a Tri-R aerosol exposure apparatus with strain P3193 Sendai virus [1–3 plaque-forming units (PFU)/ml gas] for 15 min as described (4, 6). On the study day (0, 1, 2, and 3 days after inoculation) rats were deeply anesthetized with pentobarbital sodium and killed by exsanguination via intracardiac bleeding. Right lungs were pooled by group for poly(A)^+ RNA isolation, and left lungs were individually frozen for total RNA isolation, which were used for RT-PCR analysis. Four FC rats and four FI rats at 5 days after inoculation were processed for in situ hybridization study. Fifteen-week-old male F344 rats were used to isolate rat tracheal epithelial cells.

**Construction of subtracted cDNA libraries.** Poly(A)^+ RNA was isolated with oligo(dT) cellulose (Pharmacia, Piscataway, NJ) as described (7, 21). The contaminating genomic DNA was eliminated by DNase I treatment (Promega, Madison, WI). cDNA synthesis and SSH were performed using a PCR-Select cDNA Subtraction kit (Clontech, Palo Alto, CA) according to the manufacturer’s protocol. Two micrograms of poly(A)^+ RNA from both infected and control lung samples were used to synthesize cDNAs. cDNA samples from BI and FI rats (tester cDNAs) were subtracted by cDNA samples from BC and FC rats (driver cDNAs), respectively. Subtracted cDNAs were ligated to A/T cloning vector pT-Adv (Clontech) to construct subtracted cDNA libraries for BI and FI rats. cDNAs from BI and FI rats were subtracted by cDNA samples from BC and FC rats. cDNA samples from BI and FI rats at 5 days after virus inoculation were processed for in situ hybridization study. Fifteen-week-old male F344 rats were used to isolate rat tracheal epithelial cells.

**Screening of differentially expressed genes in BN and F344 rats.** Screening of differentially expressed mRNAs was performed using the method of cDNA library array hybridization with a PCR-Select Differential Screening kit (Clontech). Two micrograms of poly(A)^+ RNA from each group was separated on a 1.5% formaldehyde gel and blotted onto a positively charged nylon membrane (Roche, Indianapolis, IN). One microgram of pooled poly(A)^+ RNA sample from each group was separated on a 1.5% formaldehyde gel and blotted onto a positively charged nylon membrane (Roche). Northern blot analysis and in situ hybridization. Riboprobes were generated by T7 and SP6 RNA polymerase with digoxigenin (DIG)-labeled UTP (Roche Diagnostics, Indianapolis, IN). One microgram of pooled poly(A)^+ RNA sample from each group was separated on a 1.5% formaldehyde gel and blotted onto a positively charged nylon membrane (Roche). Blotting membrane was fixed and then hybridized at 68°C in DIG Easy Hyb (antisense riboprobe concentration ~100 ng/ml). The hybrid signal was detected with chemiluminescent diosidum 3-(4-methoxybenzoyl)[1,2-dioxo-3,2-(5-chloro)tricyclo[3.3.1.13,7]decane]-4-yl]phenyl phosphate (Roche Diagnostics). DIG-labeled β-actin riboprobe (Roche Diagnostics) was used as an internal control to normalize mRNA concentration. Band intensities were compared between samples by ChemiImager 4400 (Alpha Innotech, San Leandro, CA). In situ hybridization was performed as described by Uhli et al. (33). Lung sections (from FC and FI rats 5 days after virus inoculation) were incubated with DIG-labeled IP-10 antisense riboprobe at 37°C. IP-10 sense riboprobe served as a negative control in this experiment.

**Sendai virus infection in cultured rat tracheal epithelial cells.** Rat tracheal epithelial cells were isolated from F344 rats and cultured as described by Kaartinen et al. (14). After cultures had been established for 2 days, tracheal epithelial cells were inoculated with Sendai virus at a multiplicity of infection of 1 PFU/cell. Plates were kept in an incubator at 37°C and 5% CO2 for 84 h; then cells were harvested and stored at −70°C for RT-PCR analysis. Untreated tracheal epithelial cells were used as control. Assays were run in triplicate.
IFN-γ was used as a positive control for IP-10 induction in tracheal epithelial cells. Medium in the established 2-day culture of rat tracheal epithelial cells was removed and replaced with fresh rat tracheal culture medium containing various doses of rat IFN-γ (0, 10, 100, or 1,000 ng/ml; Research Diagnosis, Flanders, NJ). Plates were incubated at 37°C and 5% CO2 for 8 h. Cells were collected and stored at −70°C for RT-PCR analysis. Untreated tracheal epithelial cells served as control. Assays were run in triplicate.

IP-10 abundance in cell samples was analyzed by PCR assay. Total RNA of each cell sample was isolated with TRIzol (Life Technologies, Rockville, MD). Forward primer 5′-AACCCATGAACCCAAGTG-3′ and reverse primer 5′-TGCATGTCTAGGTTCCTGTG-3′ were used in a regular PCR assay. The housekeeping gene β-actin was used as an internal control. To relatively quantify IP-10 mRNA abundance in these samples, competitive quantitative RT-PCR assay was performed using the method described in Confirmation of positive clones by quantitative RT-PCR.

Data analysis. Mean values between groups were compared by ANOVA in SigmaStat (SigmaStat for Windows; Jandel Scientific, San Rafael, CA). Differences between individual means were analyzed by the Student-Newman-Keuls method of all pairwise multiple comparison in SigmaStat.

RESULTS

Construction of subtracted cDNA libraries and differential screening. With SSH, subtracted lung cDNA libraries were constructed for virus-inoculated BN and F344 rats at 1, 2, and 3 days after Sendai virus inoculation. PCR assay indicated that >90% of the clones in libraries carried a cDNA insert. A total of 63 clones were identified as positive from 2,406 subtracted cDNA clones by cDNA library array hybridization (Table 1).

Identification of cDNA inserts. To identify cDNA inserts in the 63 positive clones, recombinants were sequenced and analyzed by BLAST search (1). Twenty-eight (44.4%) of them were identifiable as segments of cDNAs contained in GenBank, and 35 (55.6%) were unknown and submitted to the GenBank database. However, RT-PCR analysis indicated that most of the 63 clones were false positive (no significant difference between infected and control rats). Only seven clones were confirmed as inducible by Sendai virus infection and are summarized in Table 2.

Magnitude of differential expression determined by quantitative RT-PCR. The magnitude of difference in mRNA abundance of differentially expressed EST inserts among FI, BI, BC, FC rats was further examined by quantitative RT-PCR. Semiquantitative RT-PCR analysis showed that, at 3 days after inoculation, interferon-regulatory factor 7 (IRF-7) mRNA expression was induced by Sendai virus in both BN and F344 rats. However, there was no difference in levels of expression between BI and FC rats (data not shown). Similarly, expression of rat RNA helicase induced by virus 1 (RHIV-1) was upregulated by virus infection in both BN and F344 rats at 2 days after inoculation (data not shown). Bone-expressed sequence tag 5 (Best5) was also induced in a similar manner between rat strains at 2 days after virus inoculation (data not shown). Mx1 expression was induced by virus infection as early as 2 days after virus inoculation in both BN and F344 rats. At 2 days after virus inoculation, Mx1 mRNA abundance in FI rats was 188.2% greater than that in BI rats (Fig. 1A). Expression of guanylate-binding protein-2 (GBP-2) peaked at 2 days after virus inoculation. Competitive quantitative RT-PCR indicated that GBP-2 mRNA levels in FI rats were 281.7% higher than those in BI rats at 2 days after inoculation (Fig.

Table 1. Summary of results in differential screening

<table>
<thead>
<tr>
<th>Subtracted cDNA Libraries</th>
<th>Number of Subtracted Clones Screened</th>
<th>Number of Positive Clones Identified by Array Hybridization</th>
<th>Number of Positive Clones Verified by Quantitative PCR (Clone ID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI1 (susceptible)</td>
<td>381</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>FI1 (resistant)</td>
<td>382</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>BI2 (susceptible)</td>
<td>381</td>
<td>9</td>
<td>1 (best5)</td>
</tr>
<tr>
<td>FI2 (resistant)</td>
<td>379</td>
<td>10</td>
<td>2 (GBP-2 and Mx1)</td>
</tr>
<tr>
<td>BI3 (susceptible)</td>
<td>452</td>
<td>10</td>
<td>1 (RHIV-1)</td>
</tr>
<tr>
<td>FI3 (resistant)</td>
<td>431</td>
<td>13</td>
<td>3 (IP-10 and IRF-7a)</td>
</tr>
</tbody>
</table>

BI1, BN rats at 1 day after inoculation; FI1, F344 rats at 1 day after inoculation; BI2, BN rats at 2 days after inoculation; BI3, BN rats at 3 days after inoculation; FI2, F344 rats at 2 days after inoculation; BI3, BN rats at 3 days after inoculation; FI3, F344 rats at 3 days after inoculation.

Table 2. List of differentially expressed clones in rats after virus inoculation

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Identity/Homology</th>
<th>Accession No.</th>
<th>High Expression in*</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP-10</td>
<td>rat interferon-γ-inducible protein 10</td>
<td>U22520</td>
<td>F1 (higher) and BI</td>
<td>chemokine</td>
</tr>
<tr>
<td>IRF-7a</td>
<td>mouse interferon regulator factor 7</td>
<td>AW735732f</td>
<td>F1 and BI</td>
<td>transcription factor</td>
</tr>
<tr>
<td>IRF-7b</td>
<td>mouse interferon regulator factor 7</td>
<td>AW735733f</td>
<td>F1 and BI</td>
<td>transcription factor</td>
</tr>
<tr>
<td>RHIV-1</td>
<td>human RIG-I</td>
<td>BG891739f</td>
<td>F1 and BI</td>
<td>RNA helicase</td>
</tr>
<tr>
<td>GBP-2</td>
<td>mouse guanylate-binding protein-2</td>
<td>BG891748f</td>
<td>F1 (higher) and BI</td>
<td>GTPase</td>
</tr>
<tr>
<td>Mx1</td>
<td>rat Mx1</td>
<td>X522711</td>
<td>F1 (higher) and BI</td>
<td>inhibition of virus replication</td>
</tr>
<tr>
<td>best5</td>
<td>rat best5</td>
<td>Y07704</td>
<td>F1 and BI</td>
<td>bone formation</td>
</tr>
</tbody>
</table>

*F1, virus-infected F344 rats; BI, virus-infected BN rats. aGene with functional annotation with which maximum homology is obtained using a BLAST search of the public databases. bIdentity or maximum homology in the public databases. cGenBank accession number in the public databases. dHigh expression identified in this study. eTwo different cDNA fragments homologous to mouse (IRF)-7 were identified in this study. fSequences were submitted to the GenBank database.
Although differential expression of IP-10 was initially identified at 3 days after virus inoculation, its mRNA expression was already elevated 100-fold at 2 days after inoculation in both BN and F344 rats. Quantitative RT-PCR demonstrated that IP-10 mRNA in F344 rats was 201.5% greater than that in BN rats at 2 days after virus inoculation (Fig. 1C). Virus-induced IP-10 mRNA abundance was still at high levels at 5 days after virus inoculation in both BN and F344 rats (data not shown).

IP-10 differential expression indicated by Northern blot. Differential expression of IP-10 was further verified by Northern blot. As seen in Fig. 2, IP-10 baseline expression in both BN and F344 rats was undetectable by Northern blot analysis. At 3 days after virus infection, IP-10 mRNA expression levels were dramatically upregulated in both F344 and BN rats. Comparison of band intensities normalized by β-actin indicated that FI rats had 213.0% greater IP-10 mRNA than BI rats. This result was consistent with the findings from quantitative RT-PCR assay.

Airway epithelial cells highly express IP-10 mRNA at 5 days after virus inoculation. Localization of IP-10 mRNA expression was performed by in situ hybridization. Only a very weak signal was detected in normal rat lung. However, the signal was much stronger in airway epithelial cells of F344 rats at 5 days after virus inoculation (Fig. 3). This showed that rat airway epithelial cells had been induced to express abundant IP-10 mRNA after Sendai virus infection.

Sendai virus infection can directly induce IP-10 expression in cultured tracheal epithelial cells. Increased IP-10 mRNA abundance was detected in virus-infected tracheal epithelial cells at 84 h after inoculation (Fig. 4). Competitive quantitative RT-PCR further showed that IP-10 mRNA abundance was increased 10-fold at 84 h after Sendai virus inoculation (Fig. 5). As a positive control, IFN-γ (1,000 ng/ml) increased the IP-10 mRNA abundance ~100-fold at 8 h after the addition of IFN-γ (Figs. 4 and 5). Induction of IP-10 expression was activated even at a low dose (10 ng/ml) of IFN-γ (Figs. 4 and 5).

DISCUSSION

Previous studies demonstrated that the greater susceptibilities of BN rats to Sendai virus-induced chronic airway inflammation, airway remodeling, and airway...
dysfunction were associated with low IFN-γ and high interleukin (IL)-4, TNF-α, and TGF-β compared with responses of virus-resistant F344 rats (5, 32, 33). These differences were detected by Northern blot analysis and quantitative RT-PCR between 3 and 14 days after virus inoculation. In the present study, we reasoned that gene differential expression at times between 1 and 3 days after inoculation was probably important in initiating the cascade of resistance/susceptibility genes that we had detected to date. We combined SSH and cDNA library array hybridization, which have been used successfully by others (16, 20), to identify differentially expressed mRNAs in F344 and BN rats during the early time following virus inoculation. We found that Sendai virus infection increased expression of IRF-7, Best5, RHIV-1, GBP-2, Mx1, and IP-10 in BN and F344 rats at 2 days after inoculation. IP-10, Mx1, and GBP-2 are induced by virus at higher levels in F344 rats than in BN rats. Airway epithelial cells are a major cellular source of virus-induced IP-10. Sendai virus infection directly induced IP-10 expression in the culture of rat tracheal epithelial cells. Higher virus-induced expression of IP-10 in virus-resistant F344 rats compared with virus-susceptible BN rats. IP-10 mRNA levels were measured by competitive quantitative RT-PCR. Representative assay from 3 replicates. On average, both IFN-γ and Sendai virus induced increases in IP-10 74.2- and 17.0-fold, respectively.
rats was of particular interest because of the potential role that it might play in the resistance to virus-induced airway injury and dysfunction.

IP-10 is a non-ELR CXC chemokine (22). IP-10 participates in many disease processes with two basic functions: promotion of Th1 cell responses and angiostatic activity. First, as a CXC chemokine, IP-10 can selectively recruit activated T cells, monocytes, and natural killer cells into inflammatory sites (9). T cells recruited by IP-10 are dominated by Th1 cells, since its receptor (CXCR3) is expressed at high levels on Th1 lymphocytes and at low levels on Th2 lymphocytes (23).

In addition, in vivo and in vitro studies have also indicated that IP-10 can promote expression of the Th1 cytokine IFN-γ (11, 35). Th1 responses, therefore, are selectively enhanced in IP-10-dominant inflammatory sites. In this study, we found that the IP-10 gene was expressed to higher levels in infected F344 rats than in BN rats at the early time after virus inoculation. This higher expression of IP-10 might contribute to Th1-dominant cytokine expression in infected F344 rats observed in the previous studies (5, 28). Second, IP-10 is implicated in the inhibition of endothelial cell proliferation and angiogenesis and furthers the development of remodeling (2). This inhibitory activity has been characterized extensively in idiopathic pulmonary fibrosis and bleomycin-induced pulmonary fibrosis (2, 9). Our results make it reasonable to speculate that higher expression of IP-10 in F344 rats may be an important mechanism in their resistance to Sendai virus-induced chronic airway inflammation and airway fibrosis (32, 33) through regulation of recruitment of activated T cells and related regulation of airway inflammation and repair.

In a separate series of studies (3), we tested whether blocking IP-10 early in the course of Sendai virus infection would alter the development of airway inflammation and fibrosis. F344 rats treated with a neutralizing rabbit antibody against IP-10 recruited fewer lymphocytes into lung than rats treated with normal rabbit serum at 7 days after virus inoculation (3). Neutralizing IP-10 activity resulted in a paradoxical increase in IFN-γ protein production in the lung at 7 days after inoculation (3). However, no difference in severity of chronic airway inflammation and fibrosis was associated with IP-10 neutralization. If IP-10 plays an important role in regulating susceptibility to virus-induced airway damage, it is probably a complex interaction with other cytokines such as IFN-γ.

Although IP-10 can be induced by IFN-γ in human monocytic cells (17), IP-10 is more than the downstream product of IFN-γ, since that expression of IP-10 is also induced by lipopolysaccharide, IL-1α, IFN-α, IFN-β, IL-6, and TNF-α (9) and since IP-10 can also promote Th1 responses and IFN-γ production (11, 35). Therefore, a feedback loop between IP-10 and IFN-γ could potentially influence disease processes. In this study, the possibility that differential expression of IP-10 is the result of the difference on IFN-γ expression between F344 rats and BN rats cannot be completely excluded. However, we found that Sendai virus infection in airway epithelial cells can directly induce IP-10 expression without the concurrent presence of IFN-γ.

Cultured human tracheal epithelial cells can express IL-6, IL-1β, IL-8, TNF-α, and intercellular adhesion molecule-1 in response to viral infection. (30, 31) Our study shows for the first time that cultured rat tracheal epithelial cells are induced to express IP-10 after Sendai virus inoculation.

Mx1 was identified as an INF-inducible protein that confers resistance to rhabdovirus and influenza virus (29). Previous studies of our rat model (26) showed that F344 rats have a slightly more rapid clearance of virus from lungs compared with BN rats. It is possible that early higher Mx1 protein expression in F344 rats may enhance the clearance of pulmonary virus and lessen the induction of exaggerated repair processes that lead to airway fibrosis and airway remodeling, eventually.

Mouse GBP-2 was initially identified from INF-γ-induced bone marrow-derived macrophages. Except for GTPase activity in vitro, little information on its function is available. Because the induction of GBPs is common to many cell types that respond to IFNs, it is possible that GBPs may mediate some responses induced by IFNs in inflammatory and autoimmune diseases (34).

IRF-7 is a transcription factor that regulates expression of IFN-α and RANTES (regulated upon activation, normal T cell expressed and secreted) (12, 18). RHV-1 is homologous to human RIG-I. It was evident that infection of another RNA virus, porcine reproductive and respiratory syndrome virus, could also induce expression of RNA helicase (37). Best5 has been documented primarily as an INF-inducible gene expressed during osteoblast differentiation and bone formation (13).

In conclusion, we used suppressive subtractive hybridization, cDNA library array hybridization, and quantitative RT-PCR to identify seven mRNA transcripts induced by Sendai virus infection in young F344 and BN rats in this study. Of them, IP-10, GBP-2, and Mx1 were expressed to much higher levels in infected F344 than in infected BN rats at 2 days after virus inoculation. The higher expression of these three genes might play an important role in the development of Th1 responses, rapid virus clearance, and resistance to virus-induced chronic airway inflammation and fibrosis in F344 rats following Sendai virus inoculation.

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DISCLOSURES

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