Respiratory syncytial virus (RSV) produces intense pulmonary inflammation, in part, through its ability to induce chemokine synthesis in infected airway epithelial cells. RANTES (regulated upon activation, normal T-cells expressed and secreted) is a CC chemokine which recruits and activates monocytes, lymphocytes, and eosinophils, all cell types present in the lung inflammatory infiltrate induced by RSV infection. In this study we investigated the role of reactive oxygen species in the induction of RANTES gene expression in human type II alveolar epithelial cells (A549), following RSV infection. Our results indicate that RSV infection of airway epithelial cells rapidly induces reactive oxygen species production, prior to RANTES expression, as measured by oxidation of 2',7'-dichlorofluorescein. Pretreatment of airway epithelial cells with the antioxidant butylated hydroxyanisol (BHA), as well a panel of chemically unrelated antioxidants, blocks RSV-induced RANTES gene expression and protein secretion. This effect is mediated through the ability of BHA to inhibit RSV-induced interferon regulatory factor regulatory factor binding to the RANTES promoter interferon-stimulated responsive element, that is absolutely required for inducible RANTES promoter activation. BHA inhibits de novo interferon regulator factor (IRF)-1 and -7 gene expression and protein synthesis, and IRF-3 nuclear translocation. Together, these data indicates that a redox-sensitive pathway is involved in RSV-induced IRF activation, an event necessary for RANTES gene expression.
Role of ROS in RANTES Induction

like cytokines and growth factors, and infection with certain viruses, like HIV, hepatitis B, and influenza (reviewed in Ref. 12). Changes in the level of ROS, generated in response to some of these stimuli, have been shown to modulate the expression of several genes (10). Among the different members of the chemokine family, interleukin (IL)-8 is the only one for which redox-sensitive signaling pathways have been identified (13, 14). The contribution of ROS in RANTES gene expression, as well as in other CC chemokine induction, has not been defined yet. Therefore, the purpose of this study was to investigate the effect of RSV infection on ROS generation in human airway epithelial cells and the role of ROS in RSV-induced RANTES production.

Our results indicate that RSV infection of airway epithelial cells induces ROS production, as measured by intracellular oxidation of 2′,7′-dichlorofluorescein, and that treatment of airway epithelial cells with the antioxidant butylated hydroxyanisol (BHA), as well as a panel of chemically unrelated antioxidants, blocks RSV-induced RANTES protein secretion and gene expression. This effect is mediated through the inhibition of RSV-induced interferon regulatory factor (IRF) binding to the RANTES interferon-stimulated responsive element (ISRE), an event that is absolutely required for RSV-stimulated RANTES gene transcription. In infected A549 cells, ISRE binds IRF-1, -3, and -7. IRF-1 and -7 are inducible upon RSV infection of alveolar epithelial cells and treatment with BHA inhibits their gene expression and protein synthesis. In contrast, IRF-3 is constitutively expressed and antioxidant treatment blocks its nuclear translocation. These data strongly indicate that a redox-sensitive pathway is involved in RSV-induced IRF induction and RANTES gene expression. This study provides novel insights on the role of ROS in viral-induced RANTES secretion and IRF protein activation.

Identification of the molecular mechanisms involved in RANTES gene expression is fundamental for developing strategies to modulate the inflammatory response associated with RSV infection of the lung.

EXPERIMENTAL PROCEDURES

RSV Preparation—The human Long strain of RSV (A2) was grown in Hep-2 cells and purified by centrifugation on discontinuous sucrose gradients as described elsewhere (15). The virus titer of the purified RSV pools was 8–9 log10 plaque forming units/ml using a methycellulose plaque assay. No contaminating cytokines were found in these sucrose-purified viral preparations (16). Lipopolysaccharide, assayed using the limulus hemocyanin agglutination assay, was not detected.

Virus pools were aliquoted, quick-frozen on dry ice/ethanol, and stored at −70 °C until used.

Cell Culture and Infection of Epithelial Cells with RSV—A549, human alveolar type II-like epithelial cells (ATCC, Manassas, VA), were maintained in F12K medium containing 10% (v/v) fetal bovine serum, 10 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Small alveolar epithelial cells (SAE) were obtained from Clonetics, San Diego, CA, and grown according to the manufacturer’s instructions. Cell monolayers were infected with RSV at a multiplicity of infection (m.o.i.) of 1 (unless otherwise stated), as described (17). An equivalent amount of a 20% sucrose solution was added to uninfected A549 cells, as a control. For the antioxidant experiments, cells were pretreated with the antioxidants for 1 h and then infected in the presence of the antioxidants. Since BHA was diluted in ethanol, equal amounts of ethanol were added to untreated controls, as a control. Total number of cells, cell viability, and viral replication, following antioxidant treatment, were measured by trypan blue exclusion and by plaque assay, respectively.

RANTES Enzyme-linked Immunosorbent Assay—Enzyme-immunoreactive RANTES was quantitated by a double antibody enzyme-linked immunosorbent assay kit (Duoset, R&D Systems, Minneapolis, MN) following the manufacturer’s protocol.

Northern Blot—Total RNA was extracted from control and infected A549 cells by the acid guanidium thioucyanate-phenol chloroform method (18). Twenty micrograms of RNA were fractionated on a 1.2% agarose-formaldehyde gel, transferred to nylon membranes, and hybridized to a radiolabeled RANTES, IRF-1, -3, and -7 cDNAs (RANTES cDNA plasmid was a generous gift of Dr. A. Krensky, Stanford, CA; IRF-1, -3, and -7 cDNAs were a generous gift of Dr. Lin, Lady Davis Institute for Medical Research, Montreal, Quebec, Canada), as previously described (19). Hybridization temperature for all probes was 55 °C. After washing, membranes were exposed for autoradiography using Kodak X-AR film at −70 °C, using intensifying screens. After exposure, membranes were stripped and rehybridized with a β-actin probe.

Assessment of Intracellular ROS Generation—A549 cells were grown in 96-well tissue culture plates and infected with RSV at 0.1, 0.5 and 1 multiplicity of infection (m.o.i.). At different times post-infection, cells were washed with Hank’s balanced salt solution and loaded with 10 µM DCF-DA (dichlorofluorescein diacetate) or H2O2 in Hank’s balanced salt solution medium containing 25 mM HEPES, pH 7.4, for 30 min at 37 °C. The cells were then washed twice and fluorescence intensity was determined at 485 nm excitation and 590 nm emission, using an automated fluorescence reader (Fluorocount, Hewlett-Packard Instruments, IL). For the experiments in which H2O2 was used as a stimulus for ROS production, cells were preloaded with 10 µM DCF-DA for 30 min, washed, and then fluorescence intensity was measured at different times following addition of H2O2. Measurements were performed in triplicates and results expressed as fluorescence mean ± S.D. of n = 3 independent experiments.

Plasmid Construction and Cell Transfection—A fragment of the human RANTES promoter spanning from −220 to +55 nucleotides (nt), including the +1 nt (the site designated +1) of the luciferase reporter gene vector pGL2 (Promega, Madison, WI) and denoted as pGL2-220. Site mutations of the RANTES ISRE in the context of pGL2-220 plasmid were introduced by polymerase chain reaction using the following upstream and downstream mutagenic primers (mutations in lowercases): 5′-CATATTTCAGTaaATCTaC-3′ and 3′-TATAAAAGCTTTGCGGAGCT-5′.

Logarithmically growing A549 cells were transfected in triplicate in 60-mm Petri dishes by DEAE-dextran, as previously described (20). Cells were incubated in 2 ml of HEPES-buffered Dulbecco’s modified Eagle’s medium (10 mM HEPES, pH 7.4) containing 20 µl of 60 mg/ml DEAE-dextran (Amer sham Pharmacia Biotech) premixed with 6 µg of RANTES-pGL2 plasmids and 1 µg of categamovirus-β-galactosidase internal control plasmid. After 3 h, media was removed and 0.5 ml of 10% (v/v) MeSO in phosphate-buffered saline was added to the cells for 2 min. Cells were washed with phosphate-buffered saline and cultured overnight in 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium. The next morning, cells were infected with RSV and at 24 h post-infection cells were lysed to measure independently luciferase and β-galactosidase reporter activity, as previously described (20). Luciferase activity was normalized to the internal control β-galactosidase activity. All experiments were performed in duplicate or triplicate.

Electrophoretic Mobility Shift Assay—Nuclear extracts of infected and infected A549 cells were prepared using hypotonic/nonionic detergent lysis, as previously described (20). Proteins were normalized by protein assay (Protein Reagent, Bio-Rad, Hercules, CA) and used to bind to a duplex oligonucleotide corresponding to the RANTES ISRE. The ISRE sequences is 5′-GGATCCATATTTCAGTaaCTaaaCCGT-3′. A fragment of the human RANTES promoter spanning from −1 to +15 nt (MUT). The binding buffer contained 8 mM HEPES, 80 mM NaCl, 5 mM dithiothreitol, 5 mM MgCl2, 0.5 mM EDTA, 1 µg of poly(dI- dC), and 40,000 cpm of 32P-labeled double-stranded oligonucleotide in a total volume of 20 µl. The nuclear proteins were incubated with the probe for 15 min at room temperature and then fractionated by 6% nondenaturing polyacrylamide gels (PAGE) in TBE buffer (22 mM Tris-HCl, 22 mM boric acid, 0.25 mM EDTA, pH 8). After electrophoretic separation, gels were dried and exposed for autoradiography using Kodak X-AR film at −70 °C using intensifying screens.

Microarray Immobilization Assay—Microarray purification of proteins binding to the RANTES ISRE was performed using a two-step biotinylated DNA-streptavidin capture assay (20). In this assay, duplex oligonucleotides are chemically synthesized containing 5′-biotin on a flexible linker (Genosys, The Woodlands, TX). Four hundred micrograms of 12-h infected A549 cells nuclear extracts were incubated at 4 °C for 30 min with 50 pmol of biotin ISRE, in the absence or presence of 10-fold molar excess of non-biotinylated ISRE wild type (WT) or mutated (MUT). The binding buffer contained 8 µg of poly(dI-dC) (as nonspecific competitor) and 5% (v/v) MeSO in phosphate-buffered saline was added to the sample, and incubated at 4 °C for an additional 20 min with gentle rocking. Pellets were washed twice with 500 µl of binding buffer, and the washed pellets were resuspended in 100 µl of 1 × SDS-PAGE buffer, boiled, and fractionated on a 10% SDS-polyacrylamide gel. After

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Electrophoresis separation, proteins were transferred to polyvinylidene difluoride membrane for Western blot analysis.

Western Immunoblot—Total cell lysates and cytoplasmic and nuclear proteins were prepared as previously described, fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane (20). Membranes were blocked with 5% albumin in TBS-Tween and incubated overnight with a rabbit polyclonal antibody to IRF-1, -3, and -7 (Santa Cruz Biotechnology, Santa Cruz, CA). For secondary detection, we used a horseradish-coupled anti-rabbit or anti-mouse antibody in the enhanced chemiluminescence assay (Amersham Pharmacia Biotech, Arlington Heights, IL).

Statistical Analysis—Data from experiments involving multiple samples subject to each treatment were analyzed by the Student Newman Keuls t test for multiple pairwise comparisons. Results were considered significantly different at a p value, 0.05.

RESULTS

RSV Induces Reactive Oxygen Species Formation—To determine whether RSV infection induced ROS production, A549 cells were grown to ~90% of confluency and infected with RSV. At different time points after infection, cells were loaded with the membrane-permeable compound 2',7'-DCF-DA, which is trapped intracellularly following cleavage by cellular esterases. DCF oxidation was measured by changes in mean fluorescence intensity in control versus infected cells (21, 22). When cells were infected with m.o.i. of 1, the production of ROS was detectable as early as 2 h post-infection, reaching a plateau around 4 h and declining thereafter, although the level of cellular ROS in infected cells was still higher than in control cells at 24 h post-infection (Fig. 1). When cells were infected with a lower multiplicity of infection, such as 0.1 and 0.5, the kinetic of ROS production was delayed of a few hours, reaching a plateau between 6 and 8 h post-infection, reflecting the lower number of cells infected at the earliest time points.

Antioxidants Block RSV-induced RANTES Secretion—We have recently demonstrated that RSV is a potent stimulus for RANTES production in cultured human nasal, bronchial, and alveolar epithelial cells (8, 9). In all epithelial cell types, synthesis of RANTES required replicating virus and was dose- and time-dependent, with increased steady state levels of RANTES mRNA observed between 6 and 12 h after infection (8, 9). To determine the contribution of RSV-induced ROS generation in RANTES secretion, A549 cells were infected with RSV in the absence or presence of 2% (v/v) Me₂SO, 20 mM NAC, 20 mM TMTU, and 400 μM BHA. Culture supernatants, from control and infected cells were assayed 24 h later for RANTES production by enzyme-linked immunosorbent assay. Data are expressed as mean ± S.D. of three independent experiments performed in triplicates. *, p < 0.01 relative to RSV-infected cells not treated with antioxidants.
TMTU, NAC, and BHA significantly reduced RSV-induced RANTES production (Fig. 2, panel B), suggesting that indeed inducible RANTES secretion is regulated in a redox-sensitive manner. Antioxidant treatment did not significantly affect cell viability or viral replication (data not shown).

Since BHA was the most effective compound in reducing RSV-induced RANTES secretion in both A549 and SAE cells, we selected this antioxidant to perform all the subsequent experiments. To directly confirm the ability of BHA to inhibit ROS, A549 cells were stimulated with 200 μM H₂O₂ in the absence or presence of 400 μM BHA and the amount of cellular ROS was monitored by oxidation of DCFDA. As shown in Fig. 3, H₂O₂ was able to induce a high level of ROS production, which was almost completely inhibited by treatment with BHA. These data indicate that BHA function as a potent antioxidant in alveolar epithelial cells.

**BHA Inhibits RSV-induced RANTES Gene Expression**—To determine if the reduction in RSV-induced RANTES secretion by BHA was paralleled by changes in the steady-state level of RANTES mRNA, A549 cells were infected with RSV for various lengths of time, in the absence or presence of the antioxidant, and total RNA was extracted from control and infected cells for Northern blot analysis. A small increase in RANTES mRNA expression was first detected at 6 h post-infection, with maximal induction between 12 and 24 h (Fig. 4). There was no further increase in mRNA levels at later time points (data not shown). Treatment with 400 μM BHA completely inhibited RSV-induced RANTES mRNA induction at 6 and 12 h post-infection and greatly reduced it at 24 h (Fig. 4). This dramatic change was not due to a nonspecific effect since total cell number and viability in the group treated with antioxidant were unchanged (data not shown) and levels of the housekeeping gene β-actin were not systematically reduced compared with untreated cells (Fig. 4).

Inducible RANTES gene expression is controlled at both transcriptional and post-transcriptional levels (23–25). We have previously shown that in A549 cells RSV-induced RANTES promoter activation mirrors the induction of the endogenous RANTES gene mRNA, suggesting that in alveolar epithelial cells RANTES expression, following RSV infection, is controlled mainly at the level of transcription.2 To determine whether the antioxidant effect of BHA influenced RANTES gene transcription, A549 cells were transiently transfected with a construct containing the first 220 nucleotides of the human RANTES promoter linked to the luciferase reporter gene, defined as pGL2-220. This fragment of the promoter contains all the necessary regulatory elements that drive regulated luciferase expression in A549 cells following RSV infection.2 The day after, cells were infected with RSV for 24 h in the absence or presence of BHA. Similar to what we have observed for mRNA levels, treatment with BHA almost completely abolished RSV-induced luciferase activity (Fig. 5), indicating that the antioxidant effect occurs mainly by interfering with RANTES gene transcription.

**Effects of BHA Treatment on RSV-induced Transcription Factor Activation**—We have recently investigated the promoter cis-regulatory elements and nuclear factors involved in the regulation of RANTES gene transcription following RSV infection of human airway epithelial cells. The results of that study have indicated that RSV-induced RANTES transcription requires cooperation of multiple response elements, including the ISRE.2 The ISRE is absolutely required for RSV-induced promoter activation, since its mutation completely blocks RSV-induced luciferase activity (Fig. 6). To determine if BHA-induced inhibition of RANTES transcription was due to changes in the abundance of DNA-binding proteins recognizing the RANTES ISRE, we performed electrophoretic mobility shift assays using nuclear extracts prepared from A549 cells control or infected with RSV for 12 h, in the absence or presence of BHA. As shown in Fig. 7, RSV infection induced a dramatic increase in ISRE binding, which was completely abolished by treatment with BHA.

The major components of the RSV-induced ISRE binding complex are IRF-1, -3, and -7.2 It has been previously shown that IRF-7 gene expression and protein synthesis is viral inducible, while IRF-3 is constitutively expressed and translocates to the nucleus when phosphorylated in response to a viral infection (26). We have previously shown that RSV infection of A549 cells induces de novo synthesis of IRF-1 (20). To determine if RSV infection of A549 cells induced IRF-7 synthesis and IRF-3 activation, we performed Western blot analysis of cytoplasmic and nuclear proteins extracted from A549 cells uninfected or infected for various lengths of time. As shown in Fig.

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2 A. Casola, R. P. Garofalo, H. Haeberle, T. F. Elliott, M. Jamaluddin and A. R. Brasier, J. Virol. (in press).
8, RSV infection induced de novo synthesis of IRF-7 and its nuclear translocation starting around 12 h post-infection. By contrast, IRF-3 was constitutively expressed and RSV infection induced its nuclear translocation starting around 6 h post-infection (Fig. 8). The cytoplasmic abundance of IRF-3 was lower at 12 h post-infection, compared with control cells, likely due to the combined effect of nuclear translocation and cytoplasmic proteasome-mediated degradation of the activated form (27). Antioxidant treatment of RSV-infected alveolar epithelial cells greatly reduced the nuclear abundance of IRF-1 and -7 and one of the two detectable nuclear forms of IRF-3, as shown in Fig. 9. Since the other RSV-inducible nuclear form of IRF-3 was not affected by BHA treatment, we questioned if this form was able to bind to the RANTES ISRE. For this purpose, we used a two-step microaffinity isolation/Western blot assay. In this assay, biotinylated ISRE was used to bind nuclear extracts of control and 12-h infected A549 cells (20). ISRE-binding proteins were captured by the addition of streptavidin-agarose beads, washed, and the presence of bound IRF-3 was detected by Western blot. As shown in Fig. 10, there was little detectable binding of IRF-3 in control nuclear extracts, but its abundance was greatly increased after RSV infection. BHA treatment almost completely abolished IRF-3 binding, indicating that the nuclear...
form of IRF-3 not inhibitable by antioxidant treatment is not able to bind to and therefore to transactivate the RANTES ISRE.

To determine if the reduction in nuclear abundance of IRF-1, -3, and -7 following antioxidant treatment was due to inhibition of IRF gene expression, protein synthesis, or nuclear translocation, we performed Western blot analysis of whole cell extracts prepared from control and RSV-infected A549 cells, in the absence or presence of BHA. RSV infection induced a strong increase of IRF-1 and -7 protein expression and it caused a shift in the electrophoretic mobility of one of the three detectable forms of IRF-3, an event likely due to changes in IRF-3 phosphorylation, as it has been previously described in Sendai virus-infected cells (28). BHA treatment almost completely abolished RSV-induced IRF-1 and -7 protein synthesis and IRF-3 mobility shift (Fig. 11).

Since the amount of IRF-1 and -7 protein present in the cell is dependent on their gene expression, we performed Northern blot analysis of IRF-1 and -7 mRNA following RSV infection in the absence or presence of BHA. Both IRF-1 and -7 mRNA accumulation was almost completely blocked by antioxidant treatment at 6 and 12 h post-infection and significantly reduced at 24 h (Fig. 12). On the other hand, IRF-3 mRNA level was not increased following RSV infection and was unaffected by the antioxidant treatment (data not shown). In summary, these results strongly suggest that a redox-sensitive signaling pathway is involved in IRF activation and RANTES gene expression following RSV infection of alveolar epithelial cells.

**DISCUSSION**

Under normal conditions, airway epithelial cells represent an important interface between the external environment and the host. Upon infection or injury they play an important role in initiating the mucosal immune response by producing soluble factors, like chemokines, a family of small chemotactic cytokines, which are able to recruit and activate leukocytes in a cell-type specific manner (29). The immunomodulatory activity of the airway epithelium is of particular relevance to RSV infection, since the inflammatory response triggered by RSV appears to be an essential pathogenic component of RSV-induced lung damage (30). RANTES is a CC chemokine highly chemoattractant for T lymphocytes, monocytes, eosinophils, and basophils, all cell types which are present or activated in the inflammatory infiltrate that follows RSV infection of the lung. RANTES concentrations are elevated in nasal washes and bronchoalveolar lavages of children infected with RSV (6, 7) and RANTES gene is strongly expressed in RSV-infected respiratory epithelial cells (8, 9), suggesting that its production by infected epithelial cells may indeed play an important role in the pathogenesis of RSV-induced airway inflammation.

Free radicals and reactive oxygen species have recently been shown to function as second messengers influencing a variety of molecular and biochemical processes, including expression of a number of genes (reviewed in Ref. 10). The results of this study demonstrate that RSV infection of alveolar epithelial cells induces ROS formation and activates a redox-sensitive signaling pathway leading to de novo synthesis of the transcription factors IRF-1 and -7 and nuclear translocation of IRF-3 events that play a fundamental role in viral-induced RANTES gene expression (31). This is the first report of increased ROS production in airway epithelial cells following RSV infection. Our data show that the kinetic of ROS generation in infected cells is quite fast and precedes RSV-induced transcription factor activation and increase in RANTES mRNA. Several studies have pointed to the ability of certain viruses, including influenza, Sendai, hepatitis B, and HIV, to
induce the formation of ROS (reviewed in Ref. 12). In most cases, the ROS generation was a consequence of the activation of professional phagocytes like monocytes and polymorphonuclear cell, similar to what we have shown for eosinophils, in which RSV infection can induce superoxide production (32). The relationship between viral-induced ROS production and molecular and biochemical processes occurring in infected cells has been more carefully investigated only for HIV. HIV-induced ROS generation has been linked to gene expression and apoptosis (12), although a role for ROS has also recently been claimed in influenza-induced transcription factor activation and gene expression (33). That RSV infection of epithelial cells could induce ROS production was suggested by a previous study by Mastronarde et al. (34), who showed that antioxidant treatment of infected cells was able to block protein synthesis and mRNA induction of interleukin-8, a pro-inflammatory chemokine that has been extensively investigated in the past few years, whose activation involves ROS-sensitive signaling pathways (13, 14). However, that study did not show directly RSV-induced ROS generation.

Since the first evidence that reactive oxygen species can serve as subcellular messengers in signal transduction pathways leading to modification of gene transcription, there has been an explosion in the number of genes whose expression has been reported to be influenced by cellular redox changes. To date, there was only one report indicating a possible role of ROS in RANTES gene expression, where antioxidant treatment of mesangial cells stimulated with aggregated immunoglobulins, which can enhance ROS formation, was able to inhibit RANTES mRNA induction (35). However, the mechanism of RANTES inhibition by the antioxidant treatment was not investigated. In this study we were able to show that pretreatment of RSV-infected airway epithelial cells with a panel of chemically unrelated antioxidants can effectively inhibit RANTES secretion, mRNA induction, and transcription, confirming the involvement of ROS in RANTES gene expression. Although in this study we did not identify which species of ROS are specifically induced in alveolar epithelial cells infected with RSV, they probably do not include nitric oxide. Indeed, we were unable to measure changes in RSV-induced RANTES secretion in cells treated with the nitric-oxide synthase inhibitor Nitro-L-arginine methyl ester (L-NAME) (data not shown). Furthermore, a similar result was reported by Mastronarde et al. (34) for RSV-induced IL-8 secretion.

IRF transcription factors have been shown to play a fundamental role in the induction of several genes involved in the immune/inflammatory response to viral infections, including interferon α and β, cytokines like IL-15, adhesion molecules, MHC I molecules, and inducible NOS (reviewed in Ref. 36). RSV has been shown to predispose to the development of asthma (2) and recurrent episodes of wheezing in asthmatic children are often precipitated by RSV infection. Increased IRF-1 expression has been recently found in airway epithelial cells of patients with asthma, but not in normal individuals or patients with chronic bronchitis (37). IRF protein binding to the ISRE of RANTES promoter is necessary for viral induction of RANTES transcription and gene expression (31). In alveolar epithelial cells IRF-1, -3, and -7 are present in the DNA-nucleoprotein complex formed on the ISRE following RSV infection and all three are involved in RSV-induced RANTES promoter activation. In this study, we show for the first time that the antioxidant treatment interferes with RSV-induced RANTES transcription by inhibition of IRF binding to the ISRE, due to multiple effects including blocking of IRF gene expression, protein synthesis, or nuclear translocation. IRF-1 and -7 mRNA and protein levels are clearly increased in A549 cells following RSV infection and greatly decreased by the treatment with BHA. The decrease of IRF-1 and -7 protein synthesis is likely the major mechanism for their reduced nuclear abundance in RSV-infected cells treated with BHA. However, it is possible that BHA also affects IRF-1 and -7 phosphorylation, which is important for their nuclear translocation and DNA binding (36). IRF-1 gene expression is induced by interferon-γ and cytokines through the activation of STAT and NF-κB transcription factors (38). Similarly, interferon-γ activates IRF-7 gene transcription through an ISRE site that binds members of the STAT family (39). BHA treatment of A549 cells did not affect RSV-induced NF-κB nuclear translocation and DNA binding (data not shown). Therefore, it is possible that BHA treatment affects RSV-induced STAT activation, leading to inhibition of both IRF-1 and -7 gene expression.

Inhibition of phosphorylation and subsequent nuclear translocation is likely the mechanism by which BHA inhibits RSV-induced IRF-3 activation. IRF-3 gene is constitutively expressed and IRF-3 protein is present in cells in multiple forms due to different levels and sites of its phosphorylation, as recently demonstrated by Servant et al. (28). They have recently shown that IRF-3 exists as two forms in unstimulated cells: form I represents nonphosphorylated IRF-3 and form II a basally phosphorylated form. Infection with Sendai virus, as well as Newcastle disease and measles viruses, all paramyxoviruses like RSV, induces the appearance of form III and IV, which represent C-terminal phosphorylation of IRF-3. The latter two forms are able to translocate to the nucleus to induce gene expression. In the case of A549 cells, we were able to detect three forms of IRF-3 in unstimulated cells. Following RSV infection, we could detect a fourth band, which is likely to represent a hyperphosphorylated form II of IRF-3, whose formation was inhibited by antioxidant treatment. This hyperphosphorylated form would migrate to the nucleus to bind RANTES ISRE and would correspond to the nuclear form of IRF-3 which disappears following BHA treatment and therefore is no longer present in the RSV-induced ISRE binding complex, as shown by microaffinity isolation assay. The signaling pathway leading to IRF-3 activation is currently unknown. Faure et al. (40) have recently shown that IRF-1 was necessary for the NOS-2 in retinal epithelial cells stimulated with lipopolysaccharide/interferon-γ and that the antioxidant pyrroolidine dithiocarbamate was able to inhibit NOS-2 induction by interfering with lipopolysaccharide/interferon-γ-induced IRF-1 activation. In the case of NOS-2, ERK and p38 MAP kinases are potential candidates as redox-sensitive signaling molecules involved in IRF-1 activation, since both kinases are important for its gene expression (40), both can be activated by H2O2 stimulation and both are inhibited by antioxidant treatment (reviewed in Ref. 41). However, this may not be the case for RSV-induced IRF activation. In fact, even if we have evidence that ERK is also involved in RSV-induced RANTES secretion, inhibitors of ERK activation do not affect RSV induced binding to the RANTES ISRE.3 This result is in agreement with the recently published observation by Servant et al. (28) showing that pharmacological inhibitors of both ERK and p38 MAP kinases did not affect IRF-3 activation by Sendai virus infection.

In summary, our study indicates that the signaling pathway leading to IRF-1 and -7 protein expression and IRF-3 activation involves one or more redox-sensitive molecules that could be different depending on the stimulus applied. Current studies are in progress to identify those signaling molecules activated by RSV infection and leading to IRF protein induction and

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3 A. Casola, personal communication.
activation and RANTES production. Identification of the molecular mechanisms involved in RSV-induced gene expression is fundamental for developing strategies to modulate the inflammatory response associated with RSV infection of the lung.

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