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Carbocisteine inhibits rhinovirus infection in human tracheal epithelial cells

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Abstract

To examine the effects of a mucolytic drug, carbocisteine, on rhinovirus (RV) infection in airways, human tracheal epithelial cells were infected with a major group rhinovirus-RV14.   RV14 infection increased virus titers and the content of cytokines in supernatants.   Carbocisteine reduced supernatant virus titers and the RNA of RV14 in the cells, the susceptibility to RV infection in the cells, and supernatant cytokine concentrations, including interleukin (IL)-6 and IL-8, after RV14 infection. Carbocisteine reduced the mRNA expression of the intercellular adhesion molecule, (ICAM)-1, the receptor for the major group of RVs.   Carbocisteine also reduced the supernatant concentrations of a soluble form of ICAM-1, the number and the fluorescence intensity of acidic endosomes in the cells before RV infection, and reduced the nuclear factor-κB activation by RV14.   Carbocisteine also reduced the supernatant virus titers of a minor group rhinovirus-RV2, although carbocisteine did not reduce the mRNA expression of a low density lipoprotein receptor, the receptor for RV2.   These results suggest that carbocisteine inhibits RV2 infection by blocking the RV RNA entry from the endosomes and inhibits RV14 infection by the same mechanism and by reducing ICAM-1.   Carbocisteine may modulate airway inflammation by reducing the production of cytokines in rhinovirus infection.

Key words: mucolytic drug, common cold, intercellular adhesion molecule, endosome
        rhinovirus

Introduction

Rhinoviruses (RVs) are the major cause of the common cold and the most common acute infectious illnesses in humans [1]. RVs are also associated with acute exacerbations of bronchial asthma [2] and chronic obstructive pulmonary disease (COPD) [3]. Several mechanisms of action have been proposed, and the manifestations of RV-induced pathogenesis are thought to be the result of virus-induced mediators of inflammation [3-5].

RV infection induces the production of cytokines, including interleukin (IL)-1, IL-6, and IL-8 [5-7]. These cytokines have pro-inflammatory effects [8] and may be related to the pathogenesis of RV infections. Mucolytic or mucoactive drugs, such as L-carbocisteine or carbocysteine lysine salt monohydrate (SCMC-Lys) are clinically used in patients with COPD and bronchial asthma in various countries including Japan and Italy [9]. SCMC-Lys reduces the concentrations of IL-6 in breath condensate of acute COPD patients [10], suggesting anti-inflammatory effects of SCMC-Lys in COPD. SCMC-Lys [11] and S-carboxymethylcysteine (S-CMC) [12] also reduce the number of inflammatory cells in airways after exposure to cigarette smoke or sulfur dioxide in rats, and inhibits neutrophil activation [13]. These findings suggest the anti-inflammatory effects of mucolytic drugs including carbocysteine. However, the effects of carbocisteine on cytokine production in airway epithelial cells by RV infections have not been studied.

Recent reports have revealed that the major group of RVs enter the cytoplasm of infected cells after binding to its intercellular receptor adhesion molecule-1 (ICAM-1) [14, 15]. The entry of RNA of a major group rhinovirus-RV14 into the cytoplasm of infected cells is thought to be mediated by the destabilization from receptor binding and by endosomal acidification [15]. Macrolide antibiotics, such as bafilomycin [16, 17] and erythromycin [7], inhibit the infection of the major group of RVs via the reduction of

ICAM-1 expression [7, 17] and via the increase in endosomal pH [7, 16].

Glucocorticoid also inhibits RV14 infection via the reduction of ICAM-1 [18].   Airway

inflammation induced by infections of viruses, including RV, is associated with the

exacerbations of COPD [3, 4].   Carbocysteine lysine salt monohydrate (SCMC-Lys)

prevents acute exacerbations of COPD [9].   However, the mechanisms other than

anti-inflammatory effects in COPD [10] are still uncertain.   On the other hand,

N-acetylcysteine, another mucolytic drug, reduces the expression of ICAM-1 in the lung

[19].   Therefore, it is conceivable that carbocisteine may modulate the function of

airway epithelial cells, including the expression of ICAM-1, and may inhibit RV

infection.   However, the effects of carbocisteine on RV infection have not been studied.

We, therefore, studied the effects of carbocisteine on RV infection in human airway

epithelial cells.   We also examined the effects of carbocisteine on the production of

ICAM-1 and cytokines, and on endosomal pH, to clarify the mechanisms responsible for

the inhibition of RV infection.

Materials and Methods

Patient characteristics

Tracheas for cell cultures were obtained after death from 25 patients (age, 64 ± 2 yr; 10 female, 15 male) under a protocol approved by our institution's Ethics Committee. The causes of death included acute myocardial infarction (n=8), malignant tumor other than lung cancer (n=8), cerebral bleeding (n=3), rupture of an aortic aneurysm (n=2), renal failure (n=2), congestive heart failure (n=1), and malignant lymphoma (n=1).

Human tracheal epithelial cell culture

Isolation and culture of the human tracheal surface epithelial cells were performed as described previously [7, 17].

Viral stocks and detection and titration of viruses

Stocks of a minor group rhinovirus – RV2 and a major group rhinovirus - RV14 were prepared from patients with common colds by infecting human embryonic fibroblast cells as described previously [7, 17].   Detection and titration of RVs were performed by observing the cytopathic effects of viruses on the fibroblast cells using previously described methods [7, 17], and the amount of specimen required to infect 50% of the fibroblast cells (tissue culture infective dose [$TCID_{50}$]) was determined.

Detection and quantification of rhinovirus RNA

Detection and quantification of RV14 RNA in human tracheal epithelial cells were performed by reverse transcription (RT)-PCR as previously described [7, 17].   In addition, to quantify RV14 RNA and GAPDH mRNA expression in the cells after RV infection, real-time quantitative RT-PCR, using the Taqman technique (Roche Molecular Diagnostic Systems), was performed as previously described [17, 20-22].   We used the

program, PrimerExpress (Applied Biosystems), to design the probe and primers based on the guidelines for the optimal performance of the PCR [7, 17].

Measurement of LDH concentration

The amount of lactate dehydrogenase (LDH) in the culture supernatants was measured with the method described by Amador et al. [23].

Effects of carbocisteine on viral infection

To examine the effects of carbocisteine on the viral titers and the cytokine content in supernatants, the mRNA expression of ICAM-1, the receptor for the major group of RVs [14], the low-density lipoprotein (LDL) receptor, the receptor for the minor group of RVs [24], and RV14 RNA in the cells, the cells were treated with 10 μM of carbocisteine or vehicle (PBS) beginning 3 days before RV infection through the end of the experiments after RV infection [17].   This time frame was chosen because the maximum concentrations of S-carboxymethylcysteine in the serum become higher than 10 μM after oral ingestion of 1500 mg of S-carboxymethylcysteine [25].   The cells were then exposed to RV2 (10^5 TCID$_{50}$ units/ml), RV14 (10^5 TCID$_{50}$ units/ml), or vehicle (Eagle's minimum essential medium) for 60 min and cultured at 33°C with rolling, as described previously [7, 17].

Study protocol

To measure the time course of viral release during the first 24 h, we used four separate cultures from the same trachea, and calculated the results from 7 different tracheas.   We collected the culture supernatants at either 1, 6, 12, or 24 h after RV14 infection.   To measure the viral titer during the 24 - 48 h after RV infection, supernatants were also collected at 48 h after RV infection.   The viral content in the supernatant is expressed as TCID$_{50}$ units per ml.

To examine the concentration-dependent effects of carbocisteine on RV infection, cells were treated with carbocisteine at concentrations ranging from 10 nM to 30 μM.

The effects of carbocisteine on the susceptibility to RV14 infection were evaluated as previously described [6, 7], using epithelial cells pretreated with carbocisteine (10 μM, 3 days) or vehicle (PBS, 3 days).   The cells were then exposed to serial 10-fold dilutions of RV14 or vehicle (Eagle's minimum essential medium) for 1 h at 33°C.   The presence of RV14 in the supernatants collected for 1-3 days after infection was determined with the human embryonic fibroblast cell assay, described above, to assess whether infection occurred at each dose of RV used.

Measurement of expression of ICAM-1 and LDL receptor

The mRNA of ICAM-1 and LDL receptor was examined with real-time RT-PCR analysis as previously described [7].   In addition, concentrations of a soluble form of ICAM-1 (sICAM-1) in culture supernatants were measured with an enzyme immunoassay (EIA).

Effects of carbocisteine on cytokine production

We measured IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α of culture supernatants by specific enzyme-linked immunosorbent assays (ELISAs) [7].

Measurement of changes in acidic endosome distribution

The fluorescence intensity of acidic endosomes in the cells was measured as previously described with a dye, LysoSensor DND-189 (Molecular Probes) [7, 17], from 100 sec before, to 300 sec after, the treatment with carbocisteine (10 μM) or a vehicle (PBS).

Isolation of nuclear extracts and electrophoretic mobility shift assays

The extraction of nuclei and electrophoretic mobility shift assays were performed as previously described [7].

Statistical analysis

Results are expressed as means ± S.E. Statistical analysis was performed using two-way repeated analysis of variance measures (ANOVA). Subsequent post-hoc analysis was made using Bonferroni's method. For all analyses, values of $p < 0.05$ were assumed to be significant. n refers to the number of donors (tracheas) from which cultured epithelial cells were used.

Results

Effects of carbocisteine on rhinovirus infection in human tracheal epithelial cells

Exposing confluent human tracheal epithelial cell monolayers to RV2 (10^5 TCID$_{50}$ units/ml) and RV14 (10^5 TCID$_{50}$ units/ml) consistently led to infection. No detectable virus was revealed at 1 h after infection. RV2 and RV14 were detected in culture medium at 6 h, and the viral content progressively increased between 6 and 24 h after infection (Figure 1A and 1B). Viral titers of supernatants collected from 1 to 2 days after infection each also contained significant levels of RV2 and RV14 (Figure 1A and 1B). The viral titer levels in supernatants increased significantly with time for the first 48 h ($p<0.05$ in each case by ANOVA).

Treatment of the cells with carbocisteine significantly decreased the viral titers of RV2 and RV14 in supernatants at 24 h and 48 h after infection (Figure 1A and 1B). Furthermore, carbocisteine inhibited RV2 and RV14 infection in a concentration-dependent manner, and the maximum effect was obtained at 10 μM and 30 μM (Figure 1C and 1D).

To determine whether RV14 infection or carbocisteine-induced cytotoxic effects on the cultured cells caused cell detachment from the tubes after the cells made a confluent sheet, we counted the cell numbers after RV14 infection and after the treatment with carbocisteine. The cell numbers were constant in the confluent epithelial cells in the control medium, and the coefficient of variation was small (7.3%; n=15). Neither RV14 infection (10^5 TCID$_{50}$ units/ml; 2 days) nor carbocisteine treatment (10 μM; 5 days) had any effect on the cell numbers (data not shown). Cell viability, assessed by trypan blue exclusion [7], was consistently >96% in the carbocisteine-treated culture. RV14 infection and carbocisteine treatment (10 μM) did not alter the amount of LDH in the supernatants. The amount of LDH in the supernatants was 29 ± 2 IU/l before RV14

infection, 30 ± 2 IU/l two days after RV14 infection (p>0.50, n=5), and 30 ± 2 IU/l after carbocisteine treatment (10 µM; 5 days) (p>0.50, n=5).

Effects of carbocisteine on viral RNA by PCR

No detectable RV14 product band was revealed before RV14 infection (data not shown). The product band of RV14 was detected at 24 h after RV14 infection (Figure 2A), and the amount of RV14 RNA in the cells was larger at 48 h compared to that at 24 h (Figure 2B). Carbocisteine (10 µM) decreased the amount of RV14 RNA in the cells at 24 h and 48 h after infection (Figure 2B). The magnitude of inhibitory effects of carbocisteine at 48 h after infection was larger than that at 24 h (Figure 2B).

Effects of carbocisteine on susceptibility to RV14 infection

Treatment of the cells with carbocisteine decreased the susceptibility of the cells to infection by RV14. The minimum dose of RV14 necessary to cause infection in the cells treated with carbocisteine (10 µM, 3 days) (2.6 ± 0.2 log $TCID_{50}$ units/ml, n=5, p<0.05) was significantly higher than that in the cells treated with a vehicle of carbocisteine (PBS) (1.8 ± 0.2 log $TCID_{50}$ units/ml) (n=5).

Effects of carbocisteine on the expression of ICAM-1

Carbocisteine inhibited baseline ICAM-1 mRNA expression in the cells before RV14 infection (Figure 3A). Carbocisteine reduced the ICAM-1 mRNA expression by more than 50% compared to that of the cells treated with a vehicle of carbocisteine (PBS) (Figure 3A). Likewise, carbocisteine significantly reduced sICAM-1 concentrations in supernatants before RV14 infection (Figure 3B). In contrast, carbocisteine did not inhibit baseline LDL receptor mRNA expression in the cells before RV14 infection (Figure 3C).

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Effects of carbocisteine on cytokine production

Carbocisteine reduced the baseline secretion of IL-6 and IL-8 for 24 h before RV14 infection compared to that in the cells treated with a vehicle of carbocisteine (PBS) (Table 1). Furthermore, the secretion of IL-6 and IL-8 increased 24 h after RV14 infection. Carbocisteine also reduced the RV14 infection-induced secretion of IL-6 and IL-8 compared to that in the cells treated with a vehicle of carbocisteine (PBS) 24 h after RV14 infection (Table 1).

On the other hand, carbocisteine inhibited the baseline secretion of IL-1β for 24 h before RV14 infection compared to that in the cells with no carbocisteine treatment (Table 1). In contrast, the secretion of IL-1β 24 h after RV14 infection did not differ from that before RV14 infection (Table 1). TNF-α was not detectable in supernatants for 24 h before RV14 infection and for 24 h after RV14 infection.

Effects of carbocisteine on the acidification of endosomes

Acidic endosomes in human tracheal epithelial cells were stained green with LysoSensor DND-189. Green fluorescence from acidic endosomes was observed in a granular pattern in the cytoplasm (data not shown), as previously described [7]. Carbocisteine decreased the number and the fluorescence intensity of acidic endosomes with green fluorescence in the cells over time (Figure 4A). The fluorescence intensity from acidic endosomes in the epithelial cells treated with carbocisteine for 300 sec was significantly reduced (Figure 4A and 4B).

NF-κB DNA binding activity in human tracheal epithelial cells

The baseline intensity of NF-κB DNA binding activity was constant, and increased activation of NF-κB DNA binding activity was present in the cells 120 min after RV14 infection (Figure 5), as previously described [7]. Carbocisteine reduced the increased activation of NF-κB as a result of RV14 infection (Figure 5).

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Discussion

In the present study, we have shown that a mucolytic drug, carbocisteine, reduced viral titers in the supernatants and viral RNA of a major group rhinovirus - RV14, in cultured human tracheal epithelial cells. Pretreatment with carbocisteine inhibited the expression of mRNA in ICAM-1, the receptor for the major group of rhinviruses (RVs) [14], as well as the supernatant concentrations of a soluble form of ICAM-1 (sICAM-1) before RV14 infection. Because the minimum dose of RV14 necessary to cause infection in the cells treated with carbocisteine was significantly higher than that in the cells treated with a vehicle of carbocisteine, carbocisteine may inhibit RV14 infection, at least partly, by reducing the production of its receptor, ICAM-1, as observed in human tracheal epithelial cells treated with dexamethasone [18] and erythromycin [7]. Furthermore, carbocisteine reduced the fluorescence intensity of acidic endosomes in the epithelial cells. The magnitude of inhibitory effects of carbocisteine on the fluorescence intensity of acidic endosomes was similar to that of bafilomycin A_1 [17] and erythromycin [7]. On the other hand, carbocisteine also reduced supernatant virus titers of a minor group rhinovirus – RV2, although carbocisteine did not reduce the mRNA expression of a low-density lipoprotein (LDL) receptor, the receptor for the minor group of RVs [24]. Carbocisteine may also inhibit RV2 and RV14 RNA entry across acidic endosomes, as demonstrated in HeLa cells and human tracheal epithelial cells treated with bafilomycin A_1 [16, 17, 26] and erythromycin [18].

Various viruses have been reported to be responsible for exacerbations of disease in patients with COPD and bronchial asthma, including RVs, the influenza virus, and respiratory syncytial (RS) virus [2-4]. Seemungal et al. reported that 64% of COPD exacerbations were associated with a cold before the onset of exacerbations [3]. Seventy-seven viruses were detected in 39% of COPD exacerbations, and 39 (58%) of these viruses were RVs. RVs are also associated with acute exacerbations in bronchial

asthma [2].   These findings suggest that RVs may be a major pathogen responsible for acute exacerbations of COPD and bronchial asthma.

Various mechanisms have been attributed to the pathogenesis of COPD and bronchial asthma exacerbations, including airway inflammation, airway edema, bronchoconstriction, and mucus hypersecretion [4].   In addition, neutrophilic and eosinophilc inflammation in the exacerbations are associated with a variety of mediators, including IL-6 and IL-8, and the production and secretion of IL-6 and IL-8 are stimulated by RV14 in airway epithelial cells, as shown in the present study as well as previous studies [5-7].   Furthermore, ICAM-1 interacts physiologically with leukocyte function-associated antigen-1, expressed on leukocytes, and thus, plays a vital role in the recruitment and migration of immune effector cells to sites of local inflammation, as observed in patients with COPD [27].   Therefore, reduced RV14 infection-induced production of IL-6 and IL-8 by carbocisteine, observed in the present study, may be associated with the modulation of airway inflammation after RV infection, and with the prevention of acute exacerbations of chronic obstructive bronchitis, as previously described [9].

In the present study, carbocisteine reduced the baseline production of cytokines, including IL-1β, IL-6, and IL-8 as well as ICAM-1 before RV14 infection.   RV14 infection increased the production of IL-6 and IL-8, and carbocisteine also reduced RV14 infection-induced production of IL-6 and IL-8.   The contents of IL-1β in supernatants did not change at 24 h after RV14 infection, and TNF-α was not detectable in supernatants at 24 h after RV14 infection, as previously reported [7, 18].   Because carbocisteine reduced the viral titer of RV14 in supernatants, the inhibiting effects of carbocisteine on RV14 infection, and on cytokines production itself, might be associated with the reduced production of IL-6 and IL-8 in the cells treated with carbocisteine after RV14 infection.

Increased activation of NF-κB was also apparent in cells 120 min after RV14 infection, as shown in the previous studies [5, 7], and carbocisteine inhibited this activation.   NF-κB increases the expression of genes for many cytokines, such as IL-6, IL-8, and ICAM-1 [5].   Therefore, reduction of cytokines and ICAM-1 might be mediated via the carbocisteine-reduced activation of NF-κB.

The endosomal pH is thought to be regulated by vacuolar H^+-ATPases [28], and ion transport across the Na^+/H^+ antiporters [28].   Inhibitors of the Na^+/H^+ antiporters 5-(N-ethyl-N-isopropyl)amiloride (EIPA) and N''-[3-(Hydroxymethyl)-5-(1H-pyrrol-1-yl)benzoyl]guanidine methonesulfonate (FR168888), as well as a vacuolar H^+-ATPase inhibitor, bafilomycin, increase endosomal pH and inhibit RV14 infection in cultured human tracheal epithelial cells [7]. Although there is no data to support this, increased endosomal pH by carbocisteine in the present study may be associated with an inhibitory effect on vacuolar H^+-ATPases or Na^+/H^+ antiporters in airway epithelial cells.

Recent reports revealed that the major group of RVs enters the cytoplasm of infected cells after binding to its receptor, ICAM-1 [14].   The entry of the RNA of a major group rhinovirus - RV14, into the cytoplasm of infected cells is thought to be mediated by the destabilization from receptor binding and by endosomal acidification [15].   The inhibitory effects of carbocisteine on infection by RV14 and its effects on endosomal pH in the present study are consistent with those of bafilomycin and erythromycin in previous studies [7, 16, 17].   In addition, the inhibitory effects of carbocisteine on ICAM-1 expression in airway epithelial cells might also associated with inhibitory effects on RV14 infection, as previously reported for the inhibitory effects of bafilomycin, erythromycin and dexamethasone [7, 17, 18].

In the present study, the inhibitory effects of carbocisteine were observed in a small range of concentrations, and there is no complete inhibition of infection.   The precise reason is uncertain.   However, the magnitude of inhibitory effects of carbocisteine on the

RV titers in supernatants was smaller than that of dexamethasone and erythromycin [7, 18]. The weak inhibitory effects of carbocisteine might have a small range of response and incomplete inhibition of infection. The magnitude of the inhibitory effects of carbocisteine on sICAM-1 concentrations in supernatants was smaller than the magnitude of the inhibitory effects of dexamethasone and erythromycin on ICAM-1 protein expression [7, 18], although the magnitude of inhibitory effects of carbocisteine on the fluorescence intensity of acidic endosomes was similar to that of bafilomycin A_1 [17] and erythromycin [7]. Therefore, smaller inhibitory effects of carbocisteine on ICAM-1 expression might be partly associated with smaller inhibition of carbocisteine on RV infection compared with the inhibitory effects of dexamethasone and erythromycin.

Carbocysteine has an anti-oxidant action by scavenging reactive oxygen intermediates [29]. In fact, carbocysteine lysine salt (SCMC-Lys) reduces the concentration of 8-isoprostane, one of prostaglandin-like compounds during proxidation of membrane phospholipids by reactive oxygen species (ROS), in breath condensate of acute COPD patients [10]. Furthermore, bafilomycin A_1, a blocker of vacuolar H^+-ATPase, reduces acidification of endosome [17] in airway epithelial cells and reduces production of ROS in alveolar macrophages [30], suggesting the relationship between anti-oxidant effects and reduced acidification of endosomes. Reduced production of inflammatory cytokines and ICAM-1 by carbocisteine, in the present study, also suggests the anti-inflammatory effects of carbocisteine, as shown in previous studies that demonstrated reduced IL-6 concentrations in breath condensate by SCMC-Lys in COPD patients [10] and reduced number of inflammatory cells in airways in rats by SCMC-Lys [11] and S-CMC [12].

In summary, this is the first report that a mucolytic drug, carbocisteine, inhibits infection by RV14 and decreases the susceptibility of cultured human tracheal epithelial cells to RV14 infection, probably through the inhibition of ICAM-1 expression and endosomal acidification. Carbocisteine also inhibited RV2 infection, probably through

the inhibition of endosomal acidification.   Carbocisteine reduced baseline and rhinovirus infection-induced release of pro-inflammatory cytokines in supernatants, such as IL-6 and IL-8.   Carbocisteine may inhibit infection by the major and minor group of RVs, and modulate inflammatory responses in the airway epithelial cells after RV infection.

Acknowledgments

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Figure legends

Figure 1. A and B: Viral titers in supernatants of human tracheal epithelial cells obtained at different times after exposure to 10^5 $TCID_{50}$ units per ml of RV14 (A) or RV2 (B) in the presence (open circles) of carbocisteine (10 μM) or vehicle of carbocisteine (PBS) (closed circles). Viral content in the supernatant is expressed as $TCID_{50}$ units per ml. Results are means + S.E. from five different tracheae. Significant differences from viral infection alone are indicated by $*p<0.05$.

C and D: Concentration-response effects of carbocisteine on the viral titers of RV14 (C) or RV2 (D) in supernatants collected during 24 to 48 h after infection. The cells were treated with carbocisteine or vehicle (control; PBS) from three days before RV14 infection until the end of the experiments after RV14 infection. Viral content in the supernatant is expressed as $TCID_{50}$ units per ml. Results are means + S.E. from five different tracheae. Significant differences from vehicle alone (control) are indicated by $*p<0.05$.
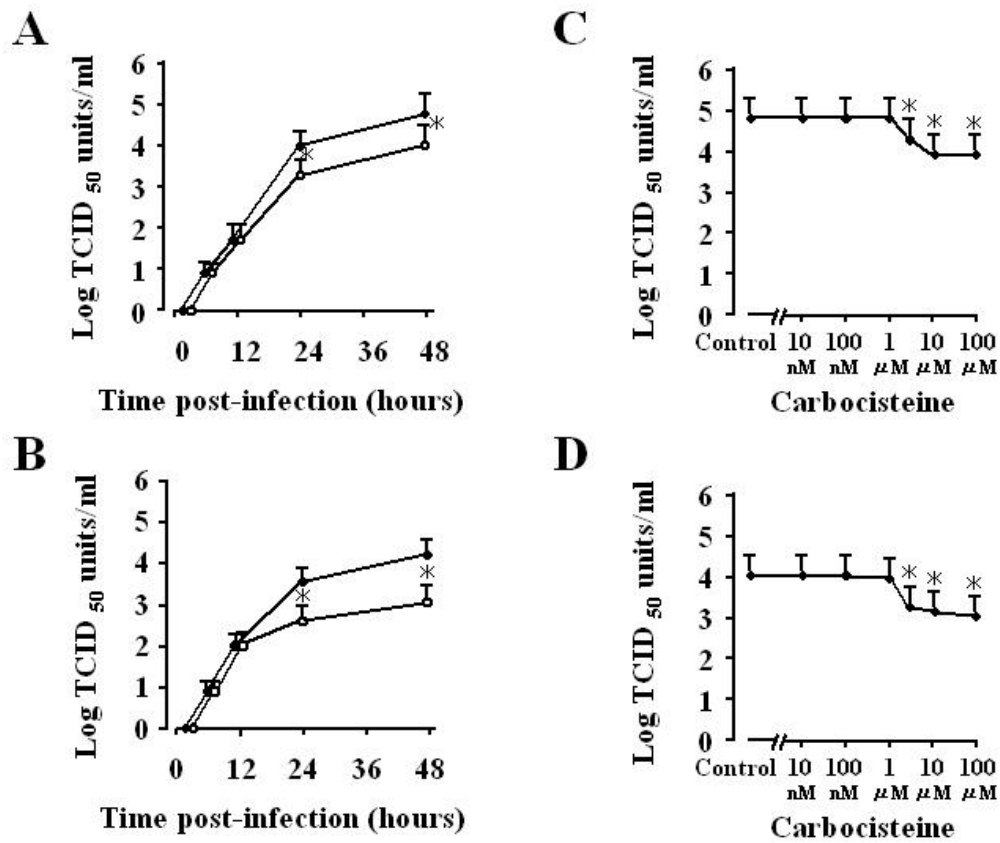
Figure 2. A: Replication of RV14 RNA from human tracheal epithelial cells at 24 and 48

h after   RV14 infection in the presence of carbocisteine (RV14 +

carbocisteine, 10 μM) or vehicle alone (RV14, PBS) as detected by RT-PCR.

Data are representative of three different experiments.

B: Replication of RV14 RNA from human tracheal epithelial cells at 24 and

48 h after   RV14 infection in the presence of carbocisteine (10 μM) (open

columns) or vehicle alone (PBS) (closed columns) as detected by real-time

quantitative RT-PCR.   A standard curve was obtained between the

fluorescence emission signals and Cτ by means of 10-fold dilutions of the

total RNA, extracted from 10^5 TCID$_{50}$ units/ml of RV14 in the supernatants

of human embryonic fibroblasts seven days after RV14 infection (10^4

TCID$_{50}$ units/ml).   Real-time quantitative RT-PCR for GAPDH was also

performed using the same PCR products.   Rhinovirus RNA expression was

normalized to the constitutive expression of GAPDH mRNA.   Results are
expressed as relative amounts of RV14 RNA expression (ratio) compared
with those of cells treated with vehicle alone (PBS) at 24 h after infection, and
represent the means + S.E. from five different tracheae.   Significant
differences from treatment with vehicle alone (RV14, a closed column) at 24
h after infection are indicated by *p<0.05 and **p<0.01.   Significant
differences from treatment with vehicle alone (RV14, a closed column) at 48
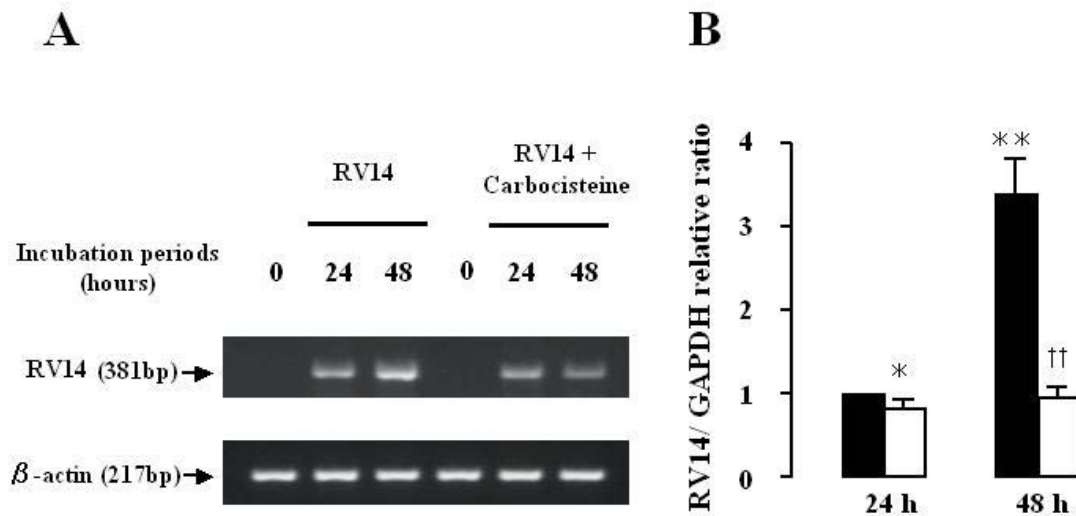h after infection are indicated by ++p<0.01.

Figure 3. A: The expression of ICAM-1 mRNA in human tracheal epithelial cells three
days after treatment with carbocisteine (Carbocisteine, 10 μM, open column) or
vehicle of carbocisteine (PBS, Control, closed column) detected by real-time

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quantitative RT-PCR.   ICAM-1 mRNA was normalized to the constitutive

expression of GAPDH mRNA.   Results are means + S.E. from five different

tracheae.   Significant differences from control values are indicated by

*p<0.05.

B: The concentrations of sICAM-1 in supernatants of the cells three days after

treatment with carbocisteine (Carbocisteine, 10 μM, open column) or vehicle

of carbocisteine (PBS, Control, closed column).   The cells were treated with

carbocisteine or vehicle of carbocisteine (PBS) for three days.   Medium was

changed every day, and the supernatants during 2-3 days after treatment with

carbocisteine or vehicle of carbocisteine were collected and the

concentrations of sICAM-1 were measured.   Results are means + S.E. from

five different tracheae.   Significant differences from control values are

indicated by *p<0.05.

C: The expression of LDL receptor (LDL-R) mRNA in human tracheal

epithelial cells 3 days after treatment with carbocisteine (Carbocisteine, 10

μM, open column) or vehicle of carbocisteine (PBS, Control, closed column)

detected by real-time quantitative RT-PCR.   LDL-R mRNA was normalized

to the constitutive expression of GAPDH mRNA.   Results are means + S.E.

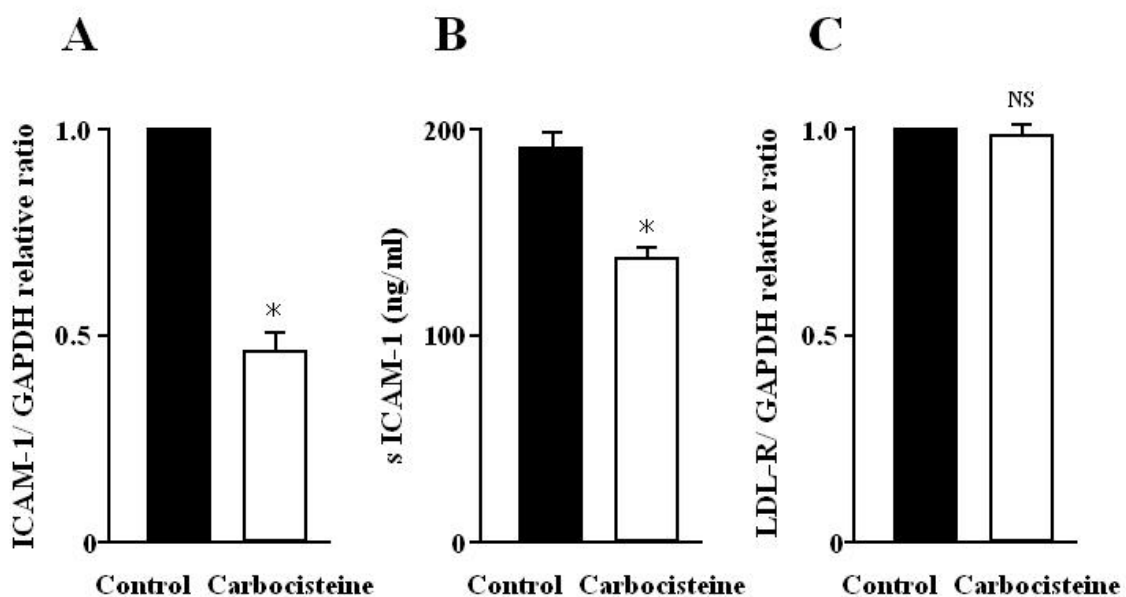from five different tracheae.   NS; not significant compared with control.

Figure 4. A: Time course changes in the intensity of green fluorescence from acidic

endosomes in human tracheal epithelial cells after treatment with either

carbocisteine (10 μM, open circles) or vehicle of carbocisteine (PBS, closed

circles).   Inhibitors were administrated at time 0.

B: The fluorescence intensity of acidic endosomes 300 sec after the addition

of carbocisteine (Carbocisteine 10 μM, open column) or vehicle of

carbocisteine (PBS, Control, closed column).   Fluorescence intensity of

acidic endosomes was measured in 100 human tracheal epithelial cells, and

the mean value of fluorescence intensity was expressed as a percentage of the

control value compared with the fluorescence intensity of the cells treated

with vehicle of carbocisteine (PBS).   Results are means + S.E. from five

A **B**
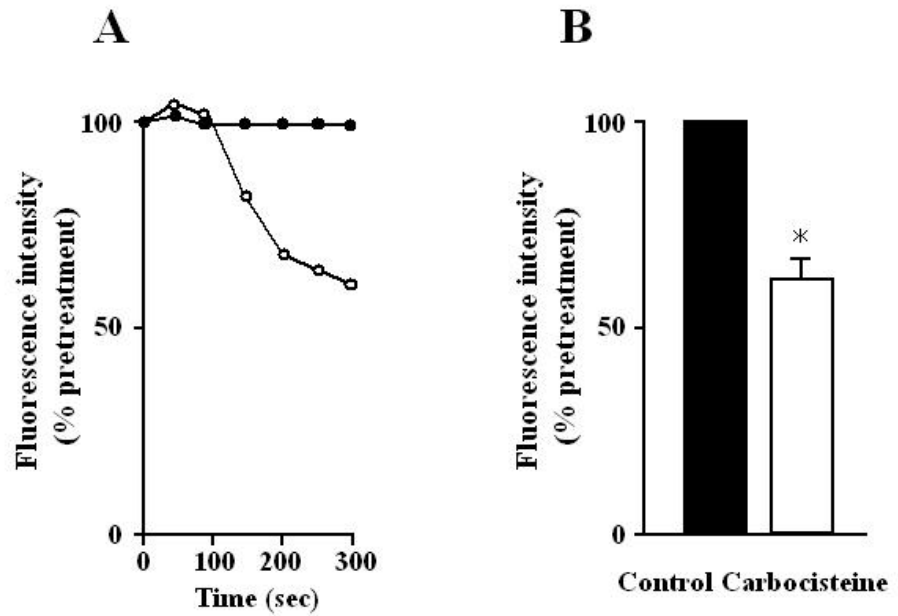
different tracheae.   Significant differences from vehicle (control) are
indicated by *p<0.05.

Figure 5. NF-κB DNA-binding activity of human tracheal epithelial cells before RV14

infection, and 120 minutes after RV14 infection in the presence of carbocisteine

[Carbocisteine (+) 10 μM] or vehicle of carbocisteine [Carbocisteine (-), PBS],

detected with electrophoreic mobility shift assay.   To examine the effects of

carbocisteine on NF-κB DNA binding activity that was stimulated after RV14

infection, the human tracheal epithelial cells were treated with carbocisteine (10

μM) or vehicle of carbocisteine (PBS) from three days before RV14 infection

until 120 min after RV14 infection.   As positive control, cells were treated with

IL-1β (10 ng/ml) plus TNF-α (10 ng/ml) for 24 hours.   Data are representative

of three different experiments.
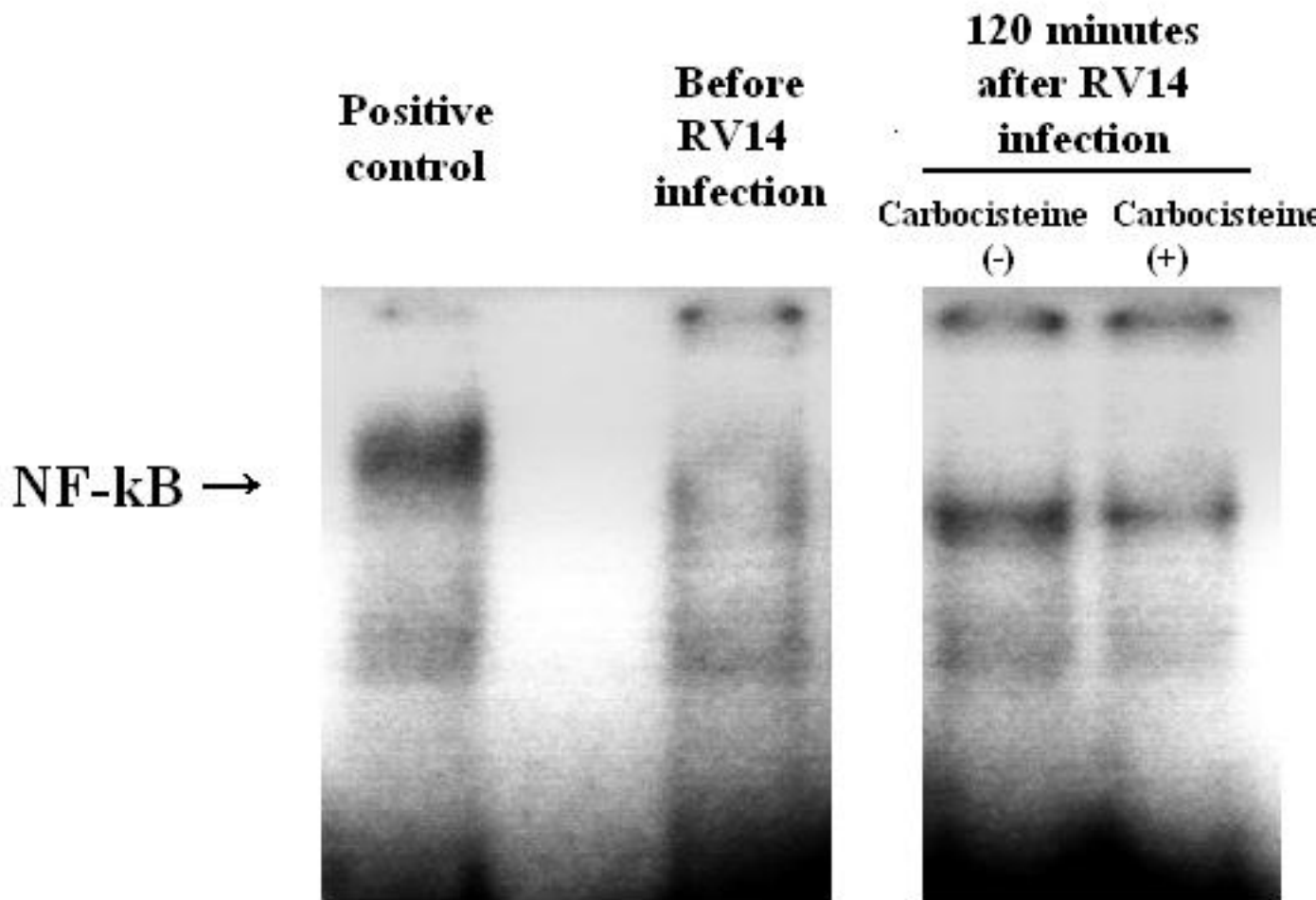
Table 1. Effects of carbocisteine on the cytokines contents in supernatants before and 24h after RV14 infection

Cytokines	Before RV14 infection		24h after RV14 infection	
	Control cells (n = 5)	Carbocisteine treated cells (n = 5)	Control cells (n = 5)	Carbocisteine treated cells (n = 5)
IL-1 β	71 ± 5	40 ± 3 *	72 ± 5	39 ± 3 *
IL-6	64 ± 4	36 ± 3 *	219 ± 23 *	123 ± 15 +*
IL-8	542 ± 41	517 ± 37 *	826 ± 44 *	491 ± 58 +*

Values are means ± S.E. *p<0.05; compared with control cells before RV14 infection.
+p<0.05; compared with control cells 24h after RV14 infection.

Control cells: human tracheal epithelial cells treated with vehicle of carbocisteine (PBS);
Carbocisteine treated cells: human tracheal epithelial cells treated with carbocisteine.

RV14: type 14 rhinovirus; IL-1 β : interleukin-1 beta; IL-6: interleukin-6; IL-8: interleukin-8.
Cytokines contents in supernatants are expressed as pg/ml.