

Rhinovirus elicits proasthmatic changes in airway responsiveness independently of viral infection

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Background: Rhinovirus (RV), the principal pathogen responsible for the common cold, is importantly implicated in triggering attacks of asthma secondary to changes in airway responsiveness.

Objective: Because the airway histopathologic features of RV infection are relatively modest, we tested the hypothesis that RV can directly elicit proasthmatic-like changes in airway smooth muscle (ASM) responsiveness independently of actual viral infection and its associated cytopathic effects.

Methods: Isolated ASM tissues and cultured ASM cells were inoculated with either infectious or noninfectious (UV-irradiated) RV16 and RV2, the latter serotypes belonging to the "major" and "minor" groups of RV subtypes, respectively. ASM constrictor and relaxant responsiveness, G_i protein expression, and proinflammatory cytokine release were subsequently compared under the different treatment conditions.

Results: In contrast to RV2, which had no effect, RV16 inoculation elicited enhanced ASM contractility and impaired relaxation to cholinergic and β -adrenergic agonists, respectively, in association with increased ASM membrane G_i protein expression and induced release of the proinflammatory cytokines IL-5 and IL-1 β . These proasthmatic-like effects were also observed in ASM exposed to UV-irradiated RV16, wherein viral replication was completely inhibited. In contrast, pretreatment of ASM with a neutralizing antibody directed against ICAM-1, the host receptor for the "major" group of RVs, completely abrogated the proasthmatic effects of RV16. **Conclusions:** The results demonstrate that (1) RV16 elicits proasthmatic changes in ASM responsiveness that can occur independently of actual viral infection of the ASM and (2) the effects of RV16 are attributed solely to binding of the virus to its host receptor (ICAM-1) on the ASM cell surface. Collectively, these findings support the notion that RV-induced exacerbation of wheezing in asthmatic individuals can occur even in the absence of any cytopathology associated with viral infection. (*J Allergy Clin Immunol* 2001;108:997-1004.)

Key words: Cytokines, transmembrane signaling, airway reactivity, common cold, asthma, intercellular adhesion molecule 1

As the principal cause of the common cold, rhinovirus (RV) infection of the respiratory tract represents the most common infectious disease in human beings. Moreover, relative to other viral respiratory pathogens, RV also represents the most common trigger of exacerbation of asthma symptoms in both children and adults.¹⁻⁵ With respect to its proasthmatic effects, there is longstanding evidence that RV exposure elicits a transient constrictor hyperreactivity of the airways, even in normal (ie, nonasthmatic) individuals.^{2,4,6} Although the fundamental mechanism(s) underlying RV-induced exacerbation of asthma symptoms and airway hyperreactivity remains to be identified, a number of studies involving the use of various cell types (eg, respiratory epithelium, respiratory submucosal glands, fibroblasts, monocytes, and macrophages) have demonstrated that exposure to RV elicits the expression and release of various proinflammatory cytokines, including IL-1 α , IL-1 β , IL-6, IL-8, IL-9, IL-11, TNF- α , and GM-CSF.⁷⁻¹⁰ As in allergic asthma, it is generally believed that the actions of these cytokines, individually or in combination, likely underlie the clinical manifestations of symptomatic RV infection. Indeed, in relating this concept to the pathogenesis of RV-induced changes in airway responsiveness, we recently determined that apart from the aforementioned cell types, RV is also capable of directly infecting airway smooth muscle (ASM) cells and eliciting their release of the T_H2-type cytokine IL-5 and the pleiotropic proinflammatory cytokine IL-1 β .^{11,12} Collectively, these findings, together with evidence that the histopathology of RV infection of the upper airway is manifested by foci of epithelial tissue damage interspersed within sections of uninvolved tissue,¹³⁻¹⁶ raise the suspicion that the symptoms of RV infection might arise from the airway immune response to the virus (eg, cytokine release) and not selectively from the sites of tissue infection and injury per se.^{8,11,17-20} In the light of this consideration, the present study examined the mechanism by which RV elicits changes in ASM responsiveness by partitioning the relative contribution of RV infection (ie, infection involving viral replication) from that specifically attributed to RV binding to the ASM cell surface. The results provide new evidence that exposure of naive ASM to RV16, a serotype representative of the "major" group of RVs, elicits proasthmatic-like changes in ASM responsiveness and proinflammatory cytokine release and that these effects occur

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Abbreviations used

ACh:	Acetylcholine
ASM:	Airway smooth muscle
ICAM-1:	Intercellular adhesion molecule 1
Rmax:	Maximal relaxation
RV:	Rhinovirus
SMBM:	Smooth muscle basal medium
Tmax:	Maximal isometric contractile force
VCAM-1:	Vascular cell adhesion molecule 1

independently of actual viral infection and induced cytopathic effects. Rather, the proasthmatic effects of RV16 are specifically attributed to binding of the virus to its endogenous host receptor, intercellular adhesion molecule 1 (ICAM-1), on the ASM cell surface.

METHODS

Animals

Twenty adult New Zealand White rabbits were used in this study, which was approved by the Biosafety and Animal Research Committee of the Joseph Stokes Research Institute at Children's Hospital of Philadelphia. The animals had no signs of respiratory disease for several weeks before study.

Culture of human RV

Stock solutions of human RV serotype 16 (RV16) and RV2 were prepared by infecting monolayer cultures of human embryonic lung fibroblasts (MRC-5) with freshly isolated RV16 and RV2 from the Clinical Virology Laboratory at the Children's Hospital of Philadelphia. As previously described,¹⁸ the cultures were grown in modified minimal essential medium supplemented with Earle's balanced salt solution, 1% L-glutamine, 7.5% FBS, HEPES buffer, and antimicrobial agents (5 µg/mL gentamicin, 10 µg/mL vancomycin, and 10 µg/mL amphotericin B). When infection was notably advanced, as evidenced by cytopathic effects, cell supernatants were harvested and frozen in aliquots (approximately 2 to 4 × 10⁶ virus particles per aliquot) at -70°C for use in the experiments described below.

RV inoculation and detection in cultured ASM cells

Human bronchial smooth muscle cells (Clonetics, San Diego, Calif) derived from 2 male donors, aged 16 and 21 years, were grown in smooth muscle basal medium (SMBM) supplemented with 5% FBS, 5 ng/mL insulin, 10 ng/mL epidermal growth factor, 2 ng/mL fibroblast growth factor, 50 ng/mL gentamicin, and 50 ng/mL amphotericin B. The standard experimental protocol involved growing the cells to confluence in the aforementioned medium and then starving the cells in unsupplemented SMBM for 24 hours, at which time the cells were exposed to nonirradiated RV16 or RV2 (approximately 1 × 10⁶ viral particles per T25 flask) and to RV16 or RV2 irradiated for 1 hour with UV light, both in the absence and in the presence of pretreatment of the cells with an IgG₁-type neutralizing mAb directed against either ICAM-1 or vascular cell adhesion molecule 1 (VCAM-1). The cells were then examined for RV mRNA expression and protein release of IL-5 and IL-1β into the cell culture medium, as described below.

Detection of infection of the ASM cells with RV16 was confirmed by RT-PCR and Southern blotting. Details pertaining to the

performance of the PCR reactions and subsequent preparation of the cDNA probes for Southern blot analysis by pooling, purifying, and sequencing the PCR reactions are as previously described.^{12,18} The PCR reactions were performed with cDNA reverse-transcribed from total RNA isolated from control (untreated) and RV16-inoculated cells through use of the following primer pairs for assessment of mRNA for both RV16 and the constitutively expressed ribosomal protein (RP) L7:

RV: 5'-primer 5'-GCACTTCTGTTTCCCC-3' and 3'-primer 5'-CGGACACCCAAAGTAG-3', the product being 380 bp
RPL7: 5'-primer 5'-AAGAGGCTCTCATTTTCCTGGCTG-3' and 3'-primer 5'-TCCGTTCTCCCATATGTTCC-3', the product being 157 bp.

In addition, RV16 and RV2 infection of the ASM cells was detected by viral plaque assays conducted on HeLa cells exposed, in separate experiments, to either supernatants or whole cell lysates of RV-inoculated ASM cells. In the plaque assays, which were conducted as previously described,²¹ monolayers of HeLa cells cultured in 12-well tissue culture plates were incubated with serial dilutions of the supernatants or cell lysates of cultured ASM cells inoculated for 24 hours with RV16 or RV2. The HeLa cell monolayers were overlaid with 1% agar in modified Eagle's medium, and viral plaques were allowed to develop at 34°C for up to 60 hours. All experiments were conducted in duplicate.

RV inoculation of ASM tissue

Our method of preparing rabbit ASM tissue has been described previously.^{11,12} In brief, after anesthesia with xylazine (10 mg/kg) and ketamine (50 mg/kg), the animals were killed with an intravenous overdose of sodium pentobarbital (130 mg/kg). The tracheae were removed, scraped of loose connective tissue and epithelium, and then divided into 6- to 8-mm ring segments. Each alternate adjacent ASM ring segment was then incubated for 24 hours at the optimal replication temperature for human RV in Dulbecco modified Eagle medium in the absence and in the presence of a maximal effective concentration of RV16 or RV2 (approximately 1 × 10⁶ viral particles per milliliter), as previously reported in our laboratory.^{12,18} The inoculation experiments were conducted in the absence and in the presence of 1 hour of pretreatment of the tissues with either anti-ICAM-1 mAb (5 µg/mL) or anti-VCAM-1 mAb (6 µg/mL). The tissues were aerated with a supplemental oxygen mixture (95% O₂/5% CO₂) throughout the incubation period; thereafter, the tissues' responsiveness to specific constrictor and relaxant agonists were compared as described below.

Pharmacodynamic studies of ASM responsiveness

After incubation of the tissue preparations, the ASM segments were suspended longitudinally in Harvard 20-mL organ baths containing modified Krebs-Ringer solution and aerated with 5% CO₂ in oxygen. A pH of 7.35 to 7.40 was maintained, and the organ bath temperature was held at 37°C. As previously described by our laboratory,^{12,18} isometric tension was measured through use of Grass FT .03 force transducers, and the signals were continuously displayed on a multichannel recorder. After equilibration in the organ baths for 45 minutes, each tissue was primed with a 1-minute exposure to 10⁻⁴ mol/L acetylcholine (ACh). Cholinergic contractility was subsequently assessed in the ASM segments by cumulative administration of ACh in final bath concentrations ranging from 10⁻¹⁰ to 10⁻³ mol/L. Thereafter, in separate studies, relaxation dose-response curves to isoproterenol (10⁻¹⁰ to 10⁻⁴ mol/L) were conducted in tissues half-maximally contracted with ACh. The initial

constrictor dose-response curves to ACh were analyzed in terms of the tissues' maximal isometric contractile force (Tmax) and sensitivity to the agonist, expressed as the negative logarithm of the concentration of ACh producing 50% of Tmax (pD50; ie, the geometric mean ED50 value). The relaxant responses to isoproterenol were analyzed in terms of percent of maximal relaxation (Rmax) from the initial level of active cholinergic contraction, and sensitivity to the relaxing agent was determined as the corresponding pD50 value associated with 50% of Rmax.

Determination of G_i protein expression

Cell surface expression of G_iα3 protein was assayed by Western blot analysis of membrane protein samples isolated from ASM tissues exposed for 24 hours to vehicle alone and to either nonirradiated or UV-irradiated RV16 or RV2. As previously described by our laboratory,¹² the ASM tissues were minced and homogenized, and nuclei and large particulates were removed by centrifugation. The supernatant was then centrifuged, the membrane pellet was resuspended, and the protein concentration was measured. Equivalent amounts (30 to 50 μg) of membrane protein were fractionated in 11% SDS-polyacrylamide gels; this was followed by transfer to nitrocellulose membranes. The membranes were then blotted overnight at room temperature in 25 mmol/L Tris HCl (pH 7.5), 150 mmol/L NaCl, and 0.05% Tergitol Nonidet P-40 containing 5% nonfat milk.¹² The rabbit polyclonal anti-G_iα3 primary antibody used was diluted 1:500 and incubated for 2 hours at room temperature. (The primary G_iα3 antibody was the generous gift of Dr David Manning of the University of Pennsylvania.) The primary and secondary antibody incubations and washes were done in 25 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.05% Nonidet P-40 containing 0.50% nonfat milk. The G_i protein levels were detected through use of enhanced chemoluminescence after a 1-hour incubation with a 1:1000 dilution of an antirabbit horseradish peroxidase-linked secondary antibody and subsequent exposure to autoradiography film. Expression levels of G_iα3 protein were quantitated by laser densitometry (Bio-Rad, Hercules, Calif).

ELISA measurement of IL-5 and IL-1β protein release

IL-5 and IL-1β protein levels were assayed in the culture media of ASM cells that were exposed for 24 hours to RV16, RV2, or vehicle alone, each in the absence and in the presence of anti-ICAM-1 mAb or anti-VCAM-1 mAb. The IL-5 and IL-1β cytokine levels were quantitatively assessed with an enzyme-specific immunoassay, as previously described by our laboratory.^{11,22} The assay was performed through use of a double-antibody sandwich strategy in which an acetylcholine-esterase Fab-conjugated IL-5- or IL-1β-specific secondary antibody is targeted to a first IL-5- or IL-1β-captured antibody. The enzymatic activity of the acetylcholine-esterase was measured spectrophotometrically, and relative to a linear standard curve, the results were used to quantify the amounts of the targeted IL-5 and IL-1β present in the cell culture media.

Statistical analysis

Unless otherwise indicated, results are expressed as mean ± SE values. Statistical analysis was performed through use of the 2-tailed Student *t* test or ANOVA with multiple comparison of means when appropriate. *P* values less than .05 were considered significant.

Reagents

The human ASM cells and SMBM were obtained from Clonetics, and the HeLa cells were obtained from the American Type Culture Collection. The RV and RPL7 primers were obtained from Integrated DNA Technologies Inc (Coralville, Iowa). The anti-ICAM-1

and anti-VCAM-1 neutralizing antibodies, the IL-5 and IL-1β ELISA kits, and the mouse antihuman IL-5 and IL-1β primary antibodies and the antimouse secondary antibodies used in the protein assay studies were purchased from R&D Systems (Minneapolis, Minn). ACh and isoproterenol were purchased from Sigma Chemical (St Louis, Mo). All drug concentrations are expressed as final bath concentrations. Isoproterenol and ACh were made fresh for each experiment and were dissolved in normal saline solution to prepare 10⁻³ mol/L stock solutions.

RESULTS

Effects of RV inoculation on ASM responsiveness

Infection of human ASM cells was examined at 0.5, 24, and 48 hours after inoculation with nonirradiated and UV-irradiated RV16 and assessed by RT-PCR and Southern blotting through use of RV16-specific primer pairs and a cDNA probe prepared by purifying and sequencing the PCR reactions (see Methods section). Relative to control (untreated) cells, infection was clearly detected in the nonirradiated RV16-exposed ASM cells at 24 and 48 hours after inoculation, whereas viral replication was essentially abolished in the cells exposed to UV-irradiated RV16 (Fig 1).

In separate studies, we also examined the infectious cytopathic plaque-forming effect of RV16 by exposing cultured HeLa cells, which are highly sensitive to the cytopathic effects of RV, to supernatants and cell lysates from ASM cells inoculated with nonirradiated and UV-irradiated RV16. In contrast to the distinct plaque-forming effect of nonirradiated RV16, UV-irradiated RV16 failed to induce plaque formation in the HeLa cells (Fig 2, A). Similar results were also obtained in comparable experiments through use of supernatants from ASM cells inoculated with another RV serotype, RV2 (Fig 2, B).

In the light of this evidence of the efficacy of UV-irradiation in inhibiting RV replication, we next investigated whether any RV-induced changes in ASM tissue agonist responsiveness are modulated by rendering the virus noninfectious with UV irradiation. Agonist-mediated airway constrictor and relaxation responses were generated in isolated rabbit ASM segments that were exposed for 24 hours to nonirradiated and UV-irradiated RV16 and RV2 both in the absence and in the presence of a monoclonal neutralizing antibody directed against ICAM-1 (anti-ICAM-1 mAb), the host receptor for the "major" group of RV subtypes that include RV16 but not for the "minor" group of RVs that include RV2. Furthermore, in comparable experiments, we also administered an anti-VCAM-1 mAb, the latter serving as a negative control. Relative to tissues incubated in control medium alone, the maximal constrictor (Tmax) responses to ACh were significantly enhanced in ASM segments that were exposed to either nonirradiated RV16 or UV-irradiated RV16 (Fig 3, A). Accordingly, the mean ± SE Tmax value amounted to 81.71 ± 11.74 g/g ASM wt in the control ASM; this compared with similarly increased Tmax values of 95.14 ± 8.08 and 97.99 ± 9.01 g/g ASM wt generated in the tissues inoculated with nonirradiated RV16

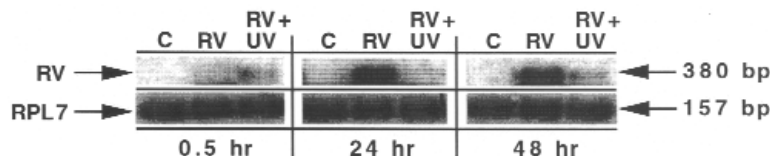


FIG 1. Comparison of Southern blots depicting RV mRNA expression in human ASM cells after 0.5, 24, and 48 hours of exposure to vehicle alone (C), nonirradiated RV16 (RV), and UV-irradiated RV16 (RV+UV). Constitutive expression of RPL7 mRNA was used to control for gel loading. Note: RV mRNA expression detected at 24 and 48 hours after inoculation of nonirradiated RV16 was ablated in cells inoculated with UV-irradiated RV16. The intensities of the corresponding RPL7 mRNA signals were essentially unaltered under all treatment conditions.

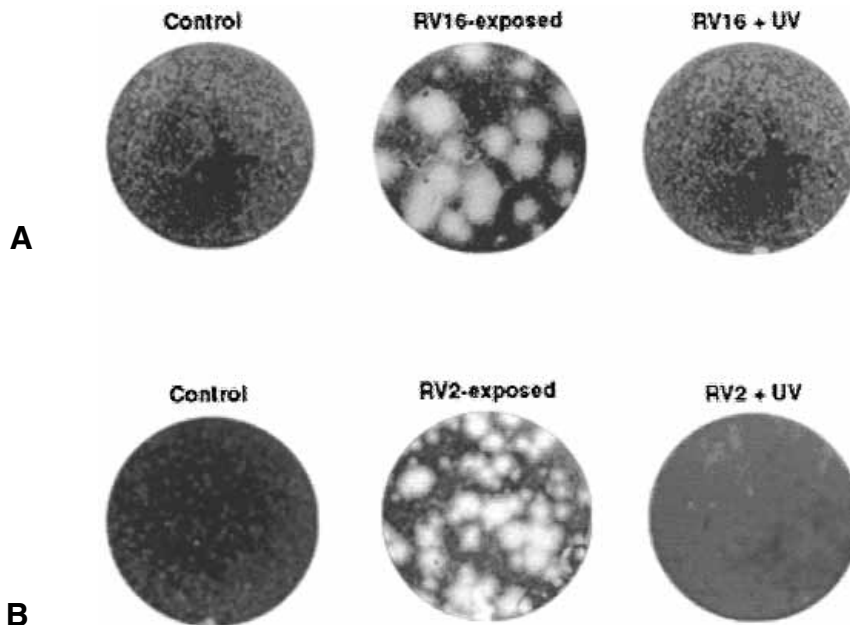


FIG 2. Comparison of viral plaque formation in HeLa cell cultures after 48 hours of exposure to supernatants from ASM cells inoculated with RV16 (A) and RV2 (B). Note: Relative to their respective non-RV-exposed controls, cultures exposed to nonirradiated RV16 and RV2 both displayed distinct viral plaque formation that was inhibited in the presence of UV-irradiated RV16 and RV2.

($P < .05$) and UV-irradiated RV16 ($P < .05$), respectively. The latter RV16-induced augmented constrictor responses to ACh were completely prevented in tissues inoculated with either nonirradiated or UV-irradiated RV16 that were pretreated with anti-ICAM-1 mAb (eg, Fig 3, A [open squares]). In contrast to these findings with RV16, inoculation of ASM tissues with either nonirradiated or UV-irradiated RV2 failed to elicit any significant changes in the tissues' constrictor responsiveness to ACh (Fig 3, B). Moreover, in extended experiments, neither pretreatment of control ASM with anti-ICAM-1 mAb nor pretreatment of either group of RV-exposed ASM with anti-VCAM-1 mAb had any appreciable effect on the tissues' constrictor responsiveness to ACh (data not shown).

In separate studies, during comparable levels of initial sustained ACh-induced contractions in control and RV-exposed segments averaging approximately 50% of T_{max} , administration of the β -adrenoceptor agonist, isoproterenol, elicited dose-dependent relaxation of the pre-

contracted tissues. Relative to control ASM, the R_{max} responses and sensitivities (pD_{50} ; ie, $-\log ED_{50}$ values) to isoproterenol were significantly attenuated in the corresponding tissues exposed to either nonirradiated RV16 or UV-irradiated RV16 (Fig 4, A). Accordingly, the mean $\pm$ SE R_{max} and pD_{50} values amounted to $52.06\% \pm 6.44\%$ and $6.37 \pm 0.05 -\log M$, respectively, in the control ASM; this compared with corresponding similarly reduced R_{max} values of $40.03\% \pm 5.36\%$ and $41.92\% \pm 6.03\%$ and attenuated pD_{50} values of 6.20 ± 0.04 and $6.19 \pm 0.04 -\log M$ obtained in the tissues inoculated with nonirradiated RV16 ($P < .01$) and UV-irradiated RV16 ($P < .01$), respectively. These RV-induced impaired relaxation responses to isoproterenol were completely abrogated in tissues inoculated with either nonirradiated or UV-irradiated RV16 that were pretreated with anti-ICAM-1 mAb (eg, Fig 4, A [open squares]), whereas the latter mAb had no effect in control ASM (data not shown). In contrast to these observations with RV16, inoculation of ASM tissues with either nonirradiated or UV-irradiated

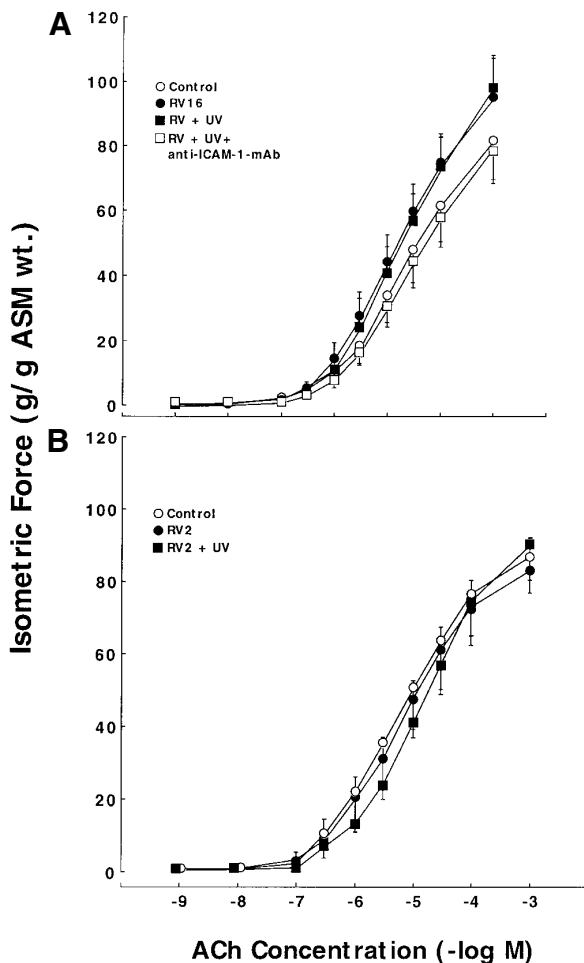


FIG 3. A, Comparison of constrictor responses to ACh in paired control (open circles) ASM segments and tissues exposed for 24 hours to nonirradiated RV16 (solid circles) and UV-irradiated RV16 in the absence (solid squares) and in the presence (open squares) of anti-ICAM-1 mAb. **B,** Comparison of paired control (open circles) ASM segments and tissues exposed to nonirradiated RV2 (solid circles) and UV-irradiated RV2 (solid squares). Note: Relative to controls, heightened constrictor responses to ACh were obtained in tissues exposed to both nonirradiated and UV-irradiated RV16, and the altered constrictor responses were prevented by pretreatment of the RV16-exposed ASM with anti-ICAM-1 mAb. In contrast, constrictor responses to ACh were similar in control and both nonirradiated and UV-irradiated RV2-exposed tissues. Data represent mean $\pm$ SE values from 6 paired experiments.

RV2 failed to induce any significant changes in the tissues' relaxant responsiveness to isoproterenol (Fig 4, B). Moreover, in contrast to anti-ICAM-1 mAb, pretreatment of either group of RV-exposed ASM with anti-VCAM-1 mAb had no effect on the tissues' relaxation responsiveness to isoproterenol (data not shown).

Effect of RV on G_i protein expression in ASM

In a previous study,¹² the effects of RV16 on ASM responsiveness reportedly were largely attributed to an induced upregulated expression and action of G_i protein (most notably, $G_{i\alpha3}$) in the RV-inoculated ASM, a con-

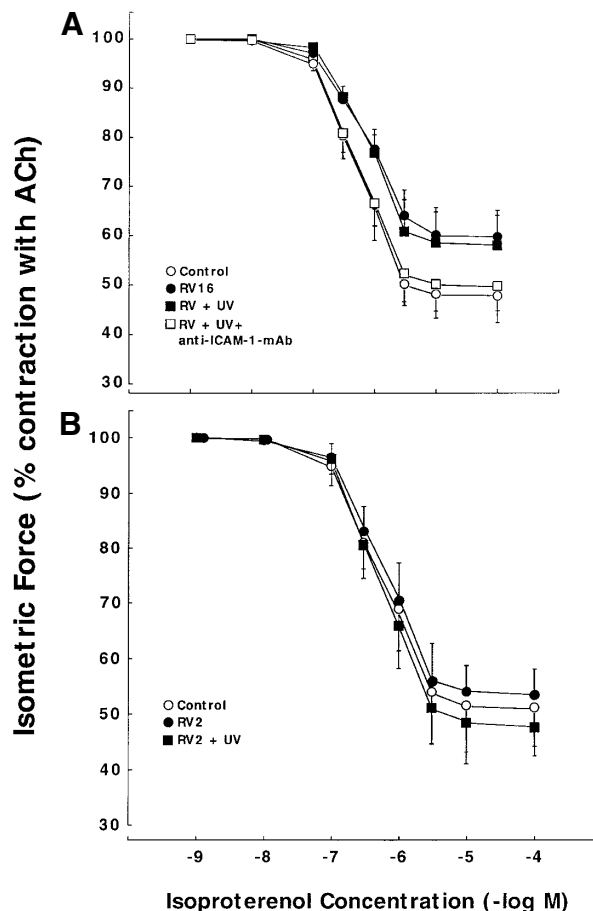


FIG 4. A, Comparison of relaxation responses to isoproterenol in paired control (open circles) ASM segments and tissues exposed for 24 hours to nonirradiated RV16 (solid circles) and UV-irradiated RV16 in the absence (solid squares) and in the presence (open squares) of anti-ICAM-1 mAb. **B,** Comparison of paired control (open circles) ASM segments and tissues exposed to nonirradiated RV2 (solid circles) and UV-irradiated RV2 (solid squares). Note: Relative to controls, similarly attenuated relaxation responses to isoproterenol were obtained in tissues exposed to nonirradiated and UV-irradiated RV16, and the attenuated relaxation responses were prevented by pretreatment of the RV16-exposed ASM with anti-ICAM-1 mAb. In contrast, relaxation responses to isoproterenol were similar in control and both nonirradiated and UV-irradiated RV2-exposed tissues. Data represent mean $\pm$ SE values from 6 paired experiments using each RV subtype.

dition associated with attenuated β -adrenoreceptor-mediated cyclic adenosine monophosphate accumulation in ASM.¹² In the light of this evidence and given the above observations, we examined whether $G_{i\alpha3}$ protein expression is differentially modulated in ASM tissues inoculated with RV2 versus RV16 by rendering the virus noninfectious with UV irradiation. Accordingly, Western immunoblot analysis of $G_{i\alpha3}$ protein expression was conducted in membrane homogenates of ASM tissues exposed for 24 hours to control (vehicle alone) medium and to nonirradiated and UV-irradiated RV2 or RV16. As indicated by the representative experiment depicted in

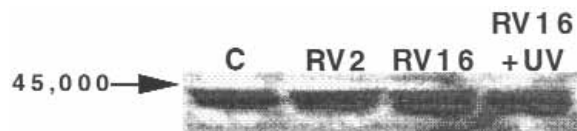


FIG 5. Comparison of Western blots of $G_i\alpha_3$ protein expression in membrane homogenates prepared from control (C), RV2-exposed (RV2), and nonirradiated (RV16) and UV-irradiated RV16-inoculated (RV16+UV) rabbit ASM tissues. Note: Relative to control, $G_i\alpha_3$ expression was similar in RV2-exposed ASM but significantly increased in tissues inoculated with either nonirradiated or UV-irradiated RV16.

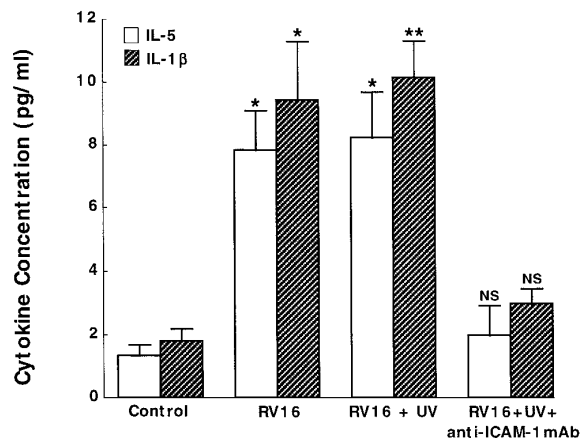


FIG 6. Comparison of IL-5 and IL-1 β protein release into the culture medium of human ASM cells after 24 hours of treatment with vehicle alone (Control) and exposure to nonirradiated RV16 (RV16) and UV-irradiated RV16 (RV16+UV) in the absence and in the presence of anti-ICAM-1 mAb. Note: Relative to control cells, release of IL-5 and IL-1 β protein was similarly significantly (* $P < .01$; ** $P < .001$) increased in cells inoculated with nonirradiated and UV-irradiated RV16, and the augmented release of these cytokines was inhibited in RV16-exposed cells pretreated with anti-ICAM-1 mAb.

Fig 5, relative to control ASM, $G_i\alpha_3$ expression was similar in RV2-exposed tissues; however, expression of $G_i\alpha_3$ was distinctly increased in RV16-inoculated ASM in both the nonirradiated and the UV-irradiated states. The $G_i\alpha_3$ protein levels quantified by laser densitometry averaged 3.1- and 3.9-fold above the corresponding control levels in the tissues exposed to nonirradiated and UV-irradiated RV16, respectively.

Effect of RV inoculation on IL-5 and IL-1 β release

We recently reported that RV16 inoculation of ASM cells induces their sequential release of IL-5 and IL-1 β , cytokines that were shown to elicit the aforementioned observed changes in ASM responsiveness and altered G_i protein expression in the RV-exposed state.^{11,12,18} Given this evidence, we next examined whether the latter reported RV16-induced release of IL-5 and IL-1 β by ASM cells is modulated by rendering the virus noninfectious with UV irradiation. In these studies, IL-5 and IL-1 β protein levels were measured by radioimmunoassay of the culture

media of human ASM cells exposed to vehicle alone (control) and to nonirradiated and UV-irradiated RV16 both in the absence and in the presence of anti-ICAM-1 mAb or anti-VCAM-1 mAb. Relative to control cells, both IL-5 and IL-1 β protein levels were significantly increased in the culture media of RV16-exposed cells, and the induced enhanced elaboration of both of these cytokines was comparable in the nonirradiated and the UV-irradiated cells (Fig 6). Moreover, this RV16-induced enhanced release of IL-5 and IL-1 β protein was virtually ablated in inoculated cells that were pretreated with anti-ICAM-1 mAb, whereas pretreatment with anti-VCAM-1 mAb had no effect (data not shown). In contrast to these results with RV16, inoculation of ASM cells with RV2 failed to induce an enhanced release of either IL-5 or IL-1 β from the cultured cells (data not shown).

DISCUSSION

In addition to eliciting upper airway symptoms associated with the common cold, RV-induced cytokine release might account for the well-established role of the virus in the exacerbation of wheezing in asthmatic individuals, a phenomenon associated with RV-induced constrictor hyperreactivity of the airways, which can occur even in normal (ie, nonasthmatic) individuals.^{2,4,6} In this regard, we recently determined that RV16 is capable of directly infecting ASM cells in vitro and elicits proasthmatic-like changes in ASM tissue responsiveness that include increased agonist-mediated constrictor hyperresponsiveness and impaired β -adrenoceptor-mediated relaxation secondary to the induced sequential release and autocrine actions of IL-5 and IL-1 β in the RV-exposed ASM itself.^{11,18} Although these findings suggest a role for the autologous induced release of the latter cytokines by RV-exposed ASM, there is no histopathologic evidence that RV inoculation of the human respiratory tract in vivo is associated with actual viral infectivity of the ASM. Thus, in the light of the findings presented above, the hypothesis is raised that exposure of ASM to RV can elicit changes in ASM responsiveness and cytokine release independent of infectivity of the tissue by the viral pathogen. The results of the present study support that latter hypothesis and, to our knowledge, are the first to demonstrate that binding of a member of the "major" group of RV subtypes (ie, RV16) to its host receptor (ie, ICAM-1) on the ASM cell surface is sufficient to produce proasthmatic-like changes in ASM responsiveness and proinflammatory cytokine release, independent of actual infection of the ASM by the virus.

The present findings provide 2 lines of evidence in support of the concept that RV-induced proasthmatic changes in ASM responsiveness represent a phenomenon that is mechanistically dependent on binding of the virus to its host cell surface receptor rather than on viral infectivity of the tissue per se. First, in this respect, the observations demonstrated that whereas inoculation with RV16 and inoculation with RV2 were both capable of producing infection associated with cytopathic effects (Fig 2), the

induction of proasthmatic changes in ASM responsiveness was elicited only by RV16; RV2 had no significant effect (Figs 3 and 4). These differences appear to be explained by the fact that in contrast to RV2, the RV16 serotype uses ICAM-1 as its host cell surface receptor and signaling via ICAM-1 has been associated with the induction of asthmatic-like changes in airway responsiveness in various animal models.^{12,18,23-28} Indeed, as demonstrated herein, the observed changes in ASM responsiveness and proinflammatory cytokine release were prevented in RV16-exposed tissues that were pretreated with an anti-ICAM-1 neutralizing antibody (Figs 3, 4, and 6).

The second line of evidence in support of the concept presented is given by our observations that though UV irradiation was efficacious in completely inhibiting RV infectivity (Figs 1 and 2), UV-irradiated RV16 remained fully capable of eliciting ICAM-1-dependent changes in ASM responsiveness and proinflammatory cytokine release (Figs 3, 4, and 6). Taken together, the present observations thus support the notion that independently of viral infection and its accompanying cytopathic effects, the ASM undergoes proasthmatic-like changes in agonist responsiveness and proinflammatory release after its exposure to the "major" group of RVs that uses ICAM-1 as the host cell surface receptor. To the extent that the present findings pertain to *in vitro* conditions involving isolated ASM, however, it remains to be established whether other cell types in the airways and/or peripheral lung tissue are capable of responding to the "minor" group of RVs (ie, serotypes that do not use ICAM-1 as their host receptor) and thereby contribute to overall changes in airway responsiveness *in vivo*.

Our observed RV16-induced proasthmatic-like changes in ASM responsiveness, including heightened constrictor responsiveness to cholinergic stimulation (Fig 3) and impaired relaxation to β -adrenoreceptor stimulation (Fig 4), are phenomena previously attributed to IL-1 β -mediated upregulated expression and action of G α _i protein, principally involving G α _{i3}, which inhibits β -adrenoreceptor-coupled cyclic adenosine monophosphate accumulation.^{12,18} Our extended present observations demonstrated that whereas RV2 exposure had no effect on ASM membrane G α _{i3} protein expression, the latter was significantly increased to a similar degree in tissues inoculated with nonirradiated and UV-irradiated RV16 (Fig 5). Mechanistically, these observations are in accord with the corresponding observed changes in ASM responsiveness, as reflected by both the lack of effect of RV2 on ASM responsiveness and the similarly induced changes in ASM responsiveness elicited by nonirradiated and UV-irradiated RV16 (Figs 3 and 4). Collectively, therefore, the present findings support the concept that ICAM-1 signaling evoked by the "major" group of RVs is coupled to induced changes in G α _{i3} protein expression and action, resulting in the observed proasthmatic-like perturbations in ASM responsiveness.

The present findings being considered further, it is relevant to note that ICAM-1 has been shown to serve a duality of cell membrane receptor functions, acting both

as the cell surface receptor for the vast majority (ie, the "major" group) of RV subtypes^{29,30} and as the endogenous counter-receptor ligand for the β ₂-integrins lymphocyte function-associated antigen 1 (ie, CD11a/CD18) and Mac-1 (ie, CD11b/CD18). In serving these roles, upregulated cell surface ICAM-1 expression has been demonstrated in RV-inoculated ASM,^{12,18} and activation of ICAM-1 has been proposed in mediating the airway eosinophilia and constrictor hyperreactivity in primate²⁸ and other animal models of allergic asthma after *in vivo* antigen challenge.^{23-26,28} Of interest, we recently determined that these dual proasthmatic-like functions of ICAM-1 are manifested in a mutually inclusive manner in ASM, wherein concomitant RV exposure and atopic sensitization act cooperatively in an ICAM-1-dependent manner to potentiate the induction of proasthmatic changes in ASM responsiveness.³¹ Moreover, we found that in mediating these dual functions of ICAM-1, both RV binding and atopic sensitization of ASM act by facilitating ICAM-1-coupled transmembrane signaling via lymphocyte function-associated antigen 1 on the surface of the ASM itself.³¹ It thus appears that both RV exposure and atopic sensitization of ASM share a common ICAM-1-coupled mechanism responsible for inducing proasthmatic changes in ASM responsiveness.

An important issue related to this collection of our present findings is that our observations on the effects of RV inoculation were based on studies conducted through use of rabbit ASM tissues and cultured human ASM cells. Although the experiments using these different preparations provided results that were largely complementary in nature, the issue of potential species differences warrants consideration. In this regard, it is relevant to note that earlier studies involving the use of atopic asthmatic serum sensitization or RV exposure to elicit proasthmatic-like changes in ASM responsiveness have reported comparable effects of these treatment conditions in rabbit and human ASM cells. Accordingly, atopic asthmatic serum sensitization was found to elicit upregulated expression of the low-affinity receptor for IgE, Fc ϵ R2 (ie, CD23), in both rabbit and human ASM cells as well as increased release of IL-1 β protein from both cell types.^{11,18,31} Moreover, RV inoculation of rabbit and human ASM cells was also found to provoke the release of IL-5 and IL-1 β from both cell types.^{11,18,31} Collectively, therefore, the findings from these earlier reports, together with those of the present study, suggest that there exists a good concordance between human and rabbit ASM cells and tissues, at least with respect to the role of RV in regulating specific cytokine release and ASM responsiveness. On the other hand, it should be noted that though RV is also capable of infecting rabbit ASM cells and inducing mRNA expression of the virus in these infected cells^{12,18}—in contrast to the present findings in human ASM cells, which depict distinct cytopathic effects of the virus (Fig 2)—we found no evidence for such comparable cytopathic effects in RV-infected rabbit ASM cells.^{12,18} Thus, whereas RV is capable of binding to and infecting both human and rabbit ASM cells, the

rabbit cells do not appear susceptible to its cytopathic effects—a feature that is consistent with the known limited efficacy of RV in inducing infectious cytopathology in nonprimate animal models.

In conclusion, the results of the present study demonstrate the following:

1. In contrast to the “minor” group of RVs, which have no effect, inoculation of ASM with a member of the “major” group of RVs (RV16) elicits proasthmatic changes in ASM responsiveness associated with enhanced G_i protein expression and proinflammatory cytokine release.
2. These effects occur secondary to transmembrane signaling elicited solely by binding of the virus to its host receptor (ICAM-1) on the ASM cell surface.
3. The effects occur independently of actual infection of the ASM by the inoculated virus.

An important implication of these findings is that RV-induced changes in airway function might be manifested independently of the development of cellular damage associated with viral infectivity. The latter notion is consistent with the known potentially significant clinical sequelae of RV exposure that occur despite the relative paucity of airway tissue damage associated with inoculation of the virus.¹³⁻¹⁶ Specifically, in relating this concept to asthma, given the present observations, the possibility is raised that the exacerbation of wheezing that occurs in asthmatic individuals after RV exposure can at times be triggered even in the absence of any overt clinical symptoms or signs typically associated with the common cold.

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